# Characterization of *Leptosphaeria maculans* races in Germany and studies on resistance of *Brassica napus* to blackleg disease

**Dissertation**

**to obtain the Ph.D. degree**

**in the international Ph.D. Program for Agricultural Sciences in Göttingen (IPAG)**

**at the Faculty of Agricultural Sciences,**

**George-August-Universität Göttingen, Germany**

**by**

**Dima Alnajar**

**born in Damascus, Syria**

**Göttingen, July 2022**

1. Name of referee: Prof. Dr. Andreas von Tiedemann
2. Name of co-referee: Prof. Dr. Anna-Katrin Mahlein

Date of disputation: 14. 09. 2022

حفر بئرٍ خير من صنعِ سلاحٍ، لا أحد على الإطلاق يعرف قيمة السلام أكثر ممن وضع بذرة في الأرض

ابن العوام، عالم في الزراعة، القرن الثاني عشر

It is better to dig a well than to make a weapon. Nobody knows the value of peace more than a person who puts a seed in the soil.

Ibn Al-Awwam, Agricultural scientist, 12th century.

# Table of content

# General introduction

## 1. Oilseed rape (*Brassica napus*)

Oilseed rape (*B. napus*) is an economically important crop worldwide (Friedt et al. 2018). The origin of this species goes back to 7,500 years ago. *B. napus* allotetraploid genome (2n= 38= AACC) derives from a natural hybridization between *B. rapa* (2n= AA) and *B. oleracea* (2n= CC), followed by a chromosome doubling (Figure 1) (Chalhoub et al. 2014). *B. napus* comes originally from the Mediterranean area and is the youngest species of the family *Brassicaceae* which includes 338 genera and 3,709 species. As a crop, *B. napus* was cultivated first in India as early as 4,000 BC (Bailey et al. 2006; Snowdon et al. 2007; Frieß et al. 2020). In Europe, domestication of *B. napus* occurred in the early middle ages (OECD, 1997).

The importance of *B. napus* is attributed to its wide range of uses (Friedt et al. 2018). It is used as cooking oil, protein source, animal feed, biofuel and has various industrial uses such as producing inks, plastic cosmetics and bio-lubricants (Canola council of Canada, 2022). In Germany, oilseed rape is mainly used for biodiesel production (rapeseed oil methyl ester). In 2019, the production of 520,000 out of 900,000 ha of German oilseed rape growing areas was used for biodiesel (FNR, 2022). Moreover, oilseed rape is an important plant for honey bees, which can produce 60-90 kg of honey from each hectare cultivated with oilseed rape (Raboanatahiry et al. 2021).

*B. napus* has many common names, such as rapeseed, colza, swede rape and canola (Orlovius, 2003). Canola is the name used to describe the double-low oil produced from cultivars developed in Canada. The oil of the double-low cultivars are healthier for human consumption due to the low erucic acid content (less than 2%), and their meal is suitable for feeding animals with low glucosinolate (< 30 mol/g) (Raboanatahiry et al. 2021). There are winter and spring types of oilseed rape and they differ mainly in their growing conditions and vernalisation requirements, where winter oilseed rape needs a prolonged cold period to be able to flower (Edwards et al. 2012; Raboanatahiry et al. 2021).

Canada was the top producer of oilseed rape in 2020/2021 making more than a quarter of the global production (27%) alone, followed by the EU (22%), China (19%) and India (12%). Total worldwide production reached 29.2 million metric tons in 2020/2021 (Figure 2) (Statista, 2022). Oilseed rape cultivation was significantly intensified throughout the last decades. Jørgensen and Heick (2021) stated that the global cultivation area of oilseed rape increased by about 48% between 1995 and 2019. The productivity per hectare improved by 21% and

the global production increased by 80% in the same time frame. However, recent heat waves in many growing regions in the world has led to a significant decrease in oilseed rape production. In the European Union, yields decreased in 2019 to a value unprecedented in the last decade, producing only 16.9 million tons; the largest reduction in several years (Figure 3). This was explained by heat and drought around oilseed rape sowing time (Zinke, 2019). In addition, the decreased effectiveness of biotic-stress control methods of oilseed rape were considered a main constraint for yield growth in Europe and Australia (Zheng et al. 2020).

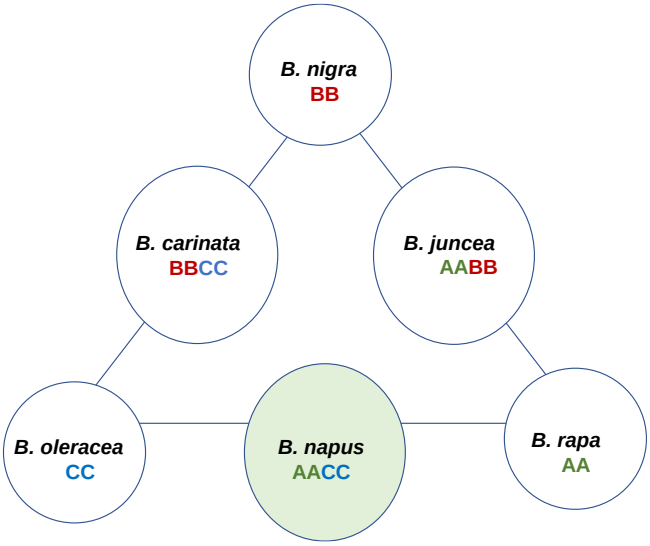



**Figure 1.** The triangle of U that shows the relationship between the tetraploid *Brassica* species and their diploid ancestors, among them the development of *Brassica napus* (Nagaharu, 1935)

A survey that covered the ten most important oilseed rape producing countries illustrates that the main biotic constraints of oilseed rape yield growth involve 16 diseases and 37 insect pests. In Europe, cabbage stem flea beetle, pollen beetle, rape stem weevil, cabbage stem weevil and *Brassica* pod midge were the most damaging insects among a list of the top ten biotic stresses, while sclerotinia stem rot, phoma stem canker, verticillium stem striping, clubroot and light leaf spot were among the most devastating diseases (Zheng et al. 2020). The importance of biotic stresses differ from region to region. While verticillium striping was among the most important diseases in Europe and Canada, it does not occur so far in Australia and China as a *B. napus* disease (Canadian Food Inspection Agency 2017; Zheng et al. 2020). Unlike in Europe, Aster yellows phytoplasma disease is a significant oilseed rape

disease in Canada (Olivier, 2012). In this study we focus on phoma stem canker, an important pathogen of winter oilseed rape in Germany.

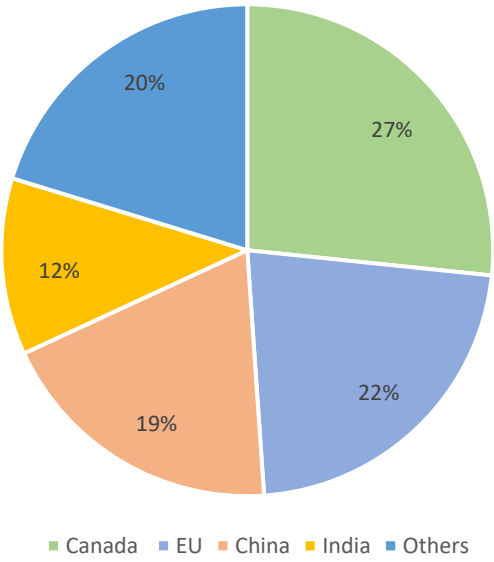



**Figure 2.** Global share of oilseed rape production (in million metric tons) in 2020/2021. Data source: Statista, 2022

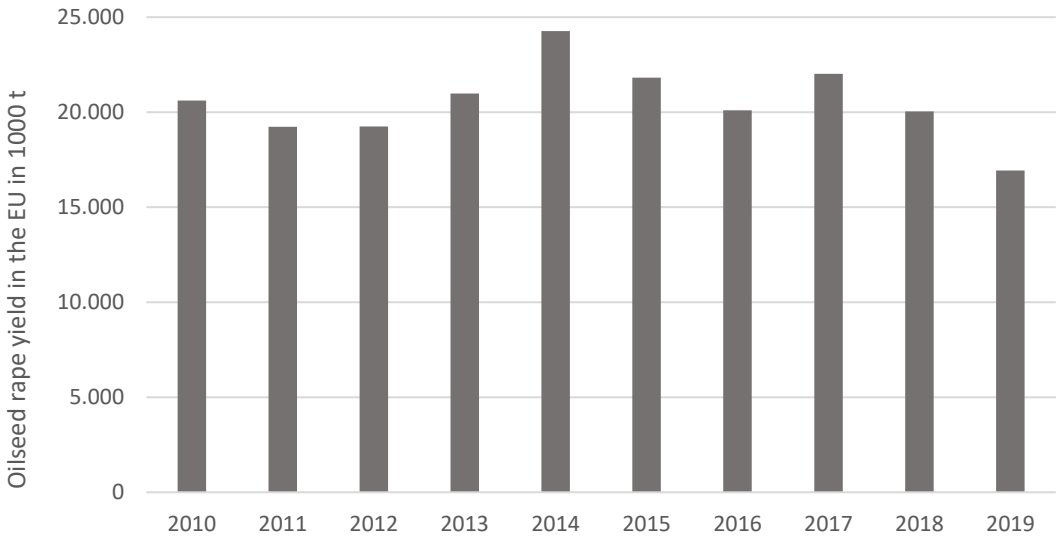



**Figure 3.** Changes in oilseed rape production in the European Union between 2010 and 2019. Data source: Agrarheute, 2019 (adapted from EU commission prognosis 2020).

## 2. Phoma stem canker (blackleg disease)

Phoma stem canker, also known as blackleg disease, is a cruciferous disease caused by two hemibiotrophic species: *L. maculans* and *L. biglobosa* (Mendes-Pereira et al. 2003). Both fungi belong to the phylum *Ascomycota*, the class *Dothideomycetes*, the order *Pleosporales*, the family *Leptosphaeriaceae* and the genus *Leptosphaeria* (Rouxel and Balesdent, 2005).

*L. maculans* and *L. biglobosa* used to be described taxonomically as two groups of a single species: the former was known as A-Group or $Tox^+$ group, while the latter was described as B-group or $Tox^-$ group. In *B. napus*, it was reported that *L. maculans* is more aggressive, produces different derivatives of the host unspecific phytotoxins sirodesmin and causes canker on oilseed rape stems and hypocotyl. *L. biglobosa*, on the other hand, is hypothesized to be less aggressive, unable to produce sirodesmins and results in mostly upper superficial stem lesions (Williams and Fitt, 1999; Rouxel and Balesdent, 2005). Both species are responsible for phoma stem canker infections in the field. However, the dominance of *L. maculans* in a population is known to be decisive for the level of disease severity (Stonard et al. 2010). *L. maculans* is usually dominant in countries where blackleg disease causes significant yield losses, whereas the disease does not seem to be of high importance in countries like China, where *L. biglobosa* was exclusively reported (Fitt et al. 2006; Liu et al. 2014). In Europe, Poland had solely *L. biglobosa* until the mid-90s. However, that has changed and it was reported that *L. maculans* has become the dominant species in Europe (Kachlicki and Jedryczka 1994; Fitt et al. 2006). As a result, *L. biglobosa* has attracted less research attention. However, there is evidence that shows that the significance of *L. biglobosa* might change again in some European countries concerning its ratio to *L. maculans* or its virulence. Fitt and Huang (2014) drew the attention to the fact that *L. biglobosa* clearly played an important role in stem canker infection in the UK in 2011/2012. Moreover, recent studies showed that some isolates of *L. biglobosa* from the UK displayed mycovirus-induced hypervirulence in comparison to other virus-free isolates due to the presence of double-stranded RNA quadriviruses (Shah et al. 2020).

*L. maculans* and *L. biglobosa* have similar complex life cycles with four trophic modes and two reproduction systems (Hammond and Lewis, 1987; Kaczmarek and Jędryczka, 2011). The fungus is a stubble-borne pathogen whose pseudothecia survive saprophytically on the residues of the previous season (Figure 3, 1). Under European conditions, pseudothecia start to mature from the beginning of September onwards. The speed of maturity is highly correlated with temperature and humidity and can vary with two-week to one-month intervals depending on the region (Dawidziuk et al. 2010). Once mature, pseudothecia eject ascospores and the primary infection occurs when ascospores germinate and hyphae grow biotrophically in the

tissues (Figure 3, 2). The symptoms on cotyledons and true leaves appear as a greyish-green collapse of tissues bearing tiny asexual fruiting bodies, the pycnidia (Figure 3, 3). In severe situations, early infection may end up with seedling damping-off in autumn (Rouxel and Balesdent, 2005). If not, pycnidia become mature and ooze a pink-coloured mass of pycnidiospores that spread to the adjacent leaves via raindrops causing secondary infections (Figure 3, 4). Hyphae develop from leaves through petioles to stems endophytically without showing any symptoms. In spring, the fungus turns necrotrophic and develops lesions on the stem and dry blackening necrosis of the crown by the end of the season (Figure 3, 5). This leads to premature plants and lodging (Figure 3, 6) (Rouxel and Balesdent, 2005). In Australia, additional untypical symptoms were observed on pods, peduncle and siliques. It was suggested to term the disease caused by *L. maculans* on these plant parts the upper canopy infection (Sprague et al. 2018). To our knowledge, there have been no reports about symptoms of upper canopy infection in Germany.

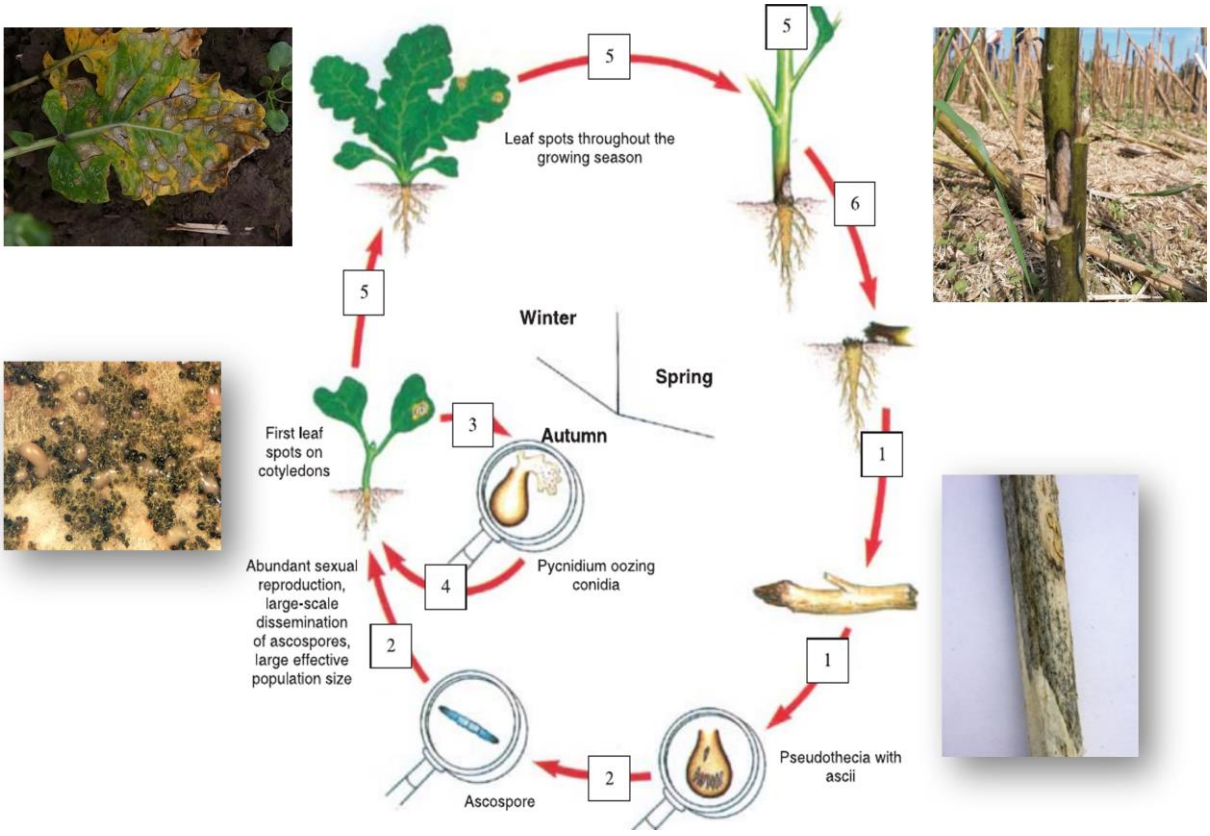



**Figure 3.** Life cycle of *L. maculans* in winter oilseed rape under European conditions (adapted from Rouxel and Balesdent, 2005)

# 3. Control methods of blackleg disease in oilseed rape

### 3.1 Cultural methods

Cultural methods mostly introduce partial control of the disease. Yet, they are indispensable to control blackleg disease in the field and participate in the success of other control methods by reducing disease pressure (Ogle and Dale, 1997). Species that belong to the cruciferous family can serve as an alternative host for blackleg. This gives weed management a sound importance. Using pathogen-free seeds, implementing a suitable crop rotation, choosing the proper sowing date are cultural control methods that considerably help manage blackleg disease (Ash, 2000). The optimal sowing date should consider regional weather conditions to define both the growth stage on which the plant shows the maximum susceptibility to infection and the period within which a significant amount of ascospores are released. Decreasing the chance that these two events overlap can help reduce disease incidence to a minimum (Aubertot et al. 2004). Nitrogen fertilization in spring did not affect phoma stem canker disease severity (Söchting and Verreet, 2004). On the contrary, adding nitrogen fertilizer in autumn during the vegetative stage increased phoma infection (Aubertot et al. 2004). Zero-tillage systems can significantly increase blackleg disease severity as the stubble - the carrier of the inoculum source, pseudothecia - remains on the soil surface. Guo et al. (2005) recommended the use of conventional tillage with a simple, one-crop rotation (e.g. oilseed rape- wheat) to reduce blackleg disease in Canada. Pseudothecia survival in the field is related to the decomposition rate of crop residues. Under conventional tillage, stubble is buried and as Naseri et al. (2008) stated, a 53.7% stubble weight loss is expected 13 months after burring stubble.

### 3.2 Fungicide use for control of blackleg disease in *B. napus*

Fungicides against blackleg disease in oilseed rape can be applied as a seed coating and/or as a foliar fungicide (West et al. 2002; Peng et al. 2020). Seed coating reduces the initial epidemic development of *L. maculans* in the sensitive seedling stage. It is, however, less effective at protecting the emerged true leaves (Peng et al. 2012). Therefore, using foliar fungicides has become the more popular method to combat the disease (Van de Wouw et al. 2021). However, foliar fungicide efficacy is temporary due to the degradation of active ingredients and the development of new leaves that have not been sprayed. This might lead farmers to spray several times and sometimes to conduct unjustified applications that increase the cost of disease control, reduce the economical reward of the crop and lead to fungicide resistance (Steed et al. 2007).

Fungicides applied against blackleg disease belong to three classes: Firstly, the quinone outside inhibitors (QoIs) that affect the mitochondrial respiratory pathway by binding to the Qo

site in the cytochrome complex bc1 (Bartlett et al. 2002; Grasso et al. 2006; Hwang et al. 2016). Secondly, the succinate dehydrogenase inhibitors (SDHIs) whose products target the mitochondrial SDH enzymes and interrupt the respiratory system (Sierotzki and Scalliet, 2013). Thirdly, the demethylation inhibitors (DMIs) to which the azole group belongs and which target the cytochrome P450 enzyme 14α-demethylase (Joseph-Horne and Hollomon, 2006; Van de Wouw et al. 2021).

The strategies of using fungicides affect their long-term efficacy and environmental side-effects. Resistance to fungicides was reported in the most important diseases of oilseed rape (Jørgensen and Heick, 2021). Monitoring for resistance against fungicides registered in Australia showed that *L. maculans* has a high level of resistance to DMIs, while resistance to SDHIs and QoIs + DMIs was rare (Van de Wouw et al. 2021). To cope with these problems, several studies aimed to develop reliable decision support systems (DSS) to rationalise the application of fungicide against blackleg disease (Gladders, 2006; Lô-Pelzer et al. 2010). Still, using cultivars that are resistant to blackleg disease seems to be the most effective and environmental friendly control method.

### 3.3 Resistant cultivars

Host resistance is the cornerstone of blackleg disease management strategies. To ease its studying, resistance in *B. napus* was classified into two types: resistance that appears early at cotyledon level; the so-called seedling resistance, and resistance whose effect can only be observed at the end of the season once the pathogen has turned necrotrophic after a long symptomless phase. Thus, it is called adult resistance or field resistance (Pilet et al. 1998; Delourme et al. 2006). Other categories were also recognised to describe these two types of resistance, such as qualitative major gene resistance and quantitative minor gene resistance. Quantitative resistance is described as partial non-race specific resistance and qualitative resistance is defined as complete race specific resistance. The former is known to be significantly affected by environmental conditions while the latter is assumed to be less affected (Delourme et al. 2006). There is no strong delineation between characters reflected in these categories. On the contrary, a clear differentiation between quantitative and qualitative resistance shown in the *L. maculans*- *B. napus* interaction is, as in many other pathosystems, rather challenging (Poland et al. 2009).

Hypersensitivity is the main mechanism of the major *R* gene mediated resistance at cotyledon level (Agrios, 2005). In this case, plant stops fungal growth through localized programmed cell death due to the gene-for-gene interaction (Flor, 1971). *R* genes encode proteins possessing a nucleotide-binding site (NBS) and leucine-rich (LRR) repeat domains (NBS-LRR proteins), which are responsible for the regulation of *R*-proteins that detect

pathogen infection through the interaction with the corresponding avirulence (*Avr*) proteins (De Young and Innes, 2006). Many major *R* genes have been commercially deployed in *B. napus* until now, such as *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7*, *Rlm9*, *LepR1*, *LepR3* and *RlmS* (Regine Delourme, pers. communication, 02.07.2018). The efficacy of these major *R* genes differs from region to region depending on the changes in the pathogen population structure. A breakdown of major *R* gene efficacy has often been reported after a boom phase (McDonald and Linde, 2002).

Quantitative resistance is known to be more durable than qualitative resistance. That is because quantitative resistance is non-race specific and thus it exerts the pathogen to less selection pressure compared to qualitative resistance (Cowger and Brown, 2019). Plenty of quantitative resistance loci (QTLs) were identified in the *B. napus* genome (reviewed in Amas et al. 2021). Nevertheless, little knowledge about its molecular mechanisms have been an obstacle for its wide deployment in commercial cultivars (Amas et al. 2021). Several mechanisms of quantitative resistance have been suggested after the success in cloning genes involved in it. For instance, numerous genes involved in encoding chemical plant defense warfare were defined (Bednarek, 2012). In addition, mutated genes associated with basal resistance or weak forms of major *R* genes shown to be involved in quantitative resistance (Raman et al. 2018). Combining quantitative resistance with major *R* genes was recommended for more durable resistance against blackleg disease in *B. napus* (Huang et al. 2018).

## 4. Aims of the study

Due to the pivotal role of resistant cultivars in controlling blackleg disease in oilseed rape, the aims of this research focus on different aspects of resistance to blackleg disease in oilseed rape:

I. Major *R* genes are widely used in commercial cultivars of *B. napus*. Intensive deployment of a major *R* gene in a specific region exposes the pathogen population to high selection pressure. This together with the high evolutionary potential of *L. maculans* leads to rapid changes in the pathogen race structure (Sprague et al. 2006). Since the efficacy of a major *R* gene is defined by the frequency of the corresponding avirulence gene in the pathogen population, regular monitoring of the pathogen and its newly emerged races in a specific region is indispensable. This provides the knowledge needed for reliable consultation regarding the use of a regionally suitable resistant cultivar. Accordingly, this study aimed to characterize the

population of *L. maculans* by their races and thereby to define the efficacy of major *R* genes in fields in Germany.

II. The observed erosion of major *R* gene efficacy in oilseed rape makes it crucial to seek new sources of resistance. Therefore, quantitative resistance has become more desirable than ever in recent years since it offers higher durability in the field. Yet, screening for quantitative resistance is challenging and needs long time under natural conditions (Amas et al. 2021). For this reason, this research aimed to establish a reliable inoculation method to efficiently monitor oilseed rape accessions for quantitative resistance against blackleg disease under controlled conditions. This method was used to phenotype plant materials of a wide genetic background, namely double haploid lines of an interconnected multiparent mapping population produced by crossing *B. napus* elite cultivars. Results were then used to define new QTLs involved in quantitative resistance to blackleg disease.

III. Beyond seeking new quantitative resistance sources in populations generated from breeding *B. napus* elite cultivars, an objective of this study was to investigate quantitative resistance resources in synthetic allohexaploid (2n= AACCBB) produced by crossing *B. napus* and *B. nigra*. The aim was to figure out if traits related to adult plant resistance were transferred from the B genome in *B. nigra* to the new allohexaploids.

IV. Finally, this research aimed to correlate quantitative resistance against blackleg disease and the occurrence of an array of phytoalexins and their dynamic changes with the development of infection in oilseed rape.

## References

Agrios, G.N. 2005. Plant pathology. 5th ed. Elsevier Academic Press, Boston, USA. ISBN 9780120445653.

Amas, J., Anderson, R., Edwards, D., Cowling, W., and Batley, J. 2021. Status and advances in mining for blackleg (*Leptosphaeria maculans*) quantitative resistance (QR) in oilseed rape (*Brassica napus*). *Theor. Appl. Genet.* 134: 3123–3145. doi:10.1007/s00122-021-03877-0.

Ash, G. 2000. Blackleg of oilseed rape. *The Plant Health Instructor APS*. doi:10.1094/PHI-I-2000-1111-01.

Aubertot, J.-N., Pinochet, X., and Doré, T. 2004. The effects of sowing date and nitrogen availability during vegetative stages on *Leptosphaeria maculans* development on winter oilseed rape. *J. Crop Prot.* 23: 635–645. doi:10.1016/j.cropro.2003.11.015.

Bailey, C.D., Koch, M.A., Mayer, M., Mummenhoff, K., O'Kane, S.L., Warwick, S.I., Windham, M.D., and Al-Shehbaz, I.A. 2006. Toward a global phylogeny of the *Brassicaceae*. *Mol. Biol. Evol.* 23: 2142–2160. doi:10.1093/molbev/msl087.

Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. *Pest. Manag. Sci.* 58: 649–662. doi:10.1002/ps.520.

Bednarek, P. 2012. Chemical warfare or modulators of defence responses – the function of secondary metabolites in plant immunity. *Curr. Opin. Plant Biol.* 15: 407–414. doi:10.1016/j.pbi.2012.03.002.

Canadian Food Inspection Agency 2017. Pest risk management document: *Verticillium longisporum* (Verticillium stripe). URL: https://inspection.canada.ca/plant-health/invasive-species/directives/pest-risk-management/rmd-17-01/eng/1487004855251/1487004951480#a64. Accessed on: 05. 05. 2022.

Canola council of Canada 2022. Grown on Canadian farms, consumed around the world. URL: https://www.canolacouncil.org/about-canola/industry/#:~:text=Grown%20on%20Canadian%20farms%2C%20consumed%20around%20the%20world&text=Canada's%20canola%20value%20chain%20is,activity%20in%20Canada%20each%20year. Accessed on: 05.05.2022

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wang, X., Chiquet, J., Belcram, H., Tong, C., Samans, B., Corréa, M., Da Silva, C., Just, J., Falentin, C., Koh, C.S., Le Clainche, I., Bernard, M., Bento, P., Noel, B…. and Wincker, P. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science* 345: 950–953. doi:10.1126/science.1253435.

Cowger, C., and Brown, J.K.M. 2019. Durability of quantitative resistance in crops: greater than we know? *Annu. Rev. Phytopathol.* 57: 253–277. doi:10.1146/annurev-phyto-082718-100016.

Dawidziuk, A., Podleśna, A., Kaczmarek, J., and Jędryczka, M. 2010. The maturation rate of the generative stage of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* in central and eastern Poland. *Polish Journal of Agronomy* (2): 3-10.

De Young, B.J., and Innes, R.W. 2006. Plant NBS-LRR proteins in pathogen sensing and host defense. *Nat. Immunol.* 7: 1243–1249. doi:10.1038/ni1410.

Delourme, R., Chèvre, A.M., Brun, H., Rouxel, T., Balesdent, M.H., Dias, J.S., Salisbury, P., Renard, M., and Rimmer, S.R. 2006. Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). in: B.D.L. Fitt, N. Evans,

B.J. Howlett, and B.M. Cooke, eds. Sustainable strategies for managing *Brassica napus* (oilseed rape) resistance to *Leptosphaeria maculans* (phoma stem canker). Springer-Verlag, Berlin/Heidelberg. 41–52, doi:10.1007/1-4020-4525-5_4.

Edwards, D., Batley, J., Parkin, I., Kole, C., 2012 (ed.) 2012. Genetics, genomics and breeding of oilseed Brassicas. 1st ed. Science Publishers ; Distributed by CRC Press, St. Helier, Jersey, British Channel Islands ; Enfield, N.H. : Boca Raton, FL. ISBN 9781578087204 .

Fitt, B., and Huang, Y. 2014. Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK. In: Conference of crop production in Southern Britain: Precision decisions for profitable cropping *in Peterborough Arena UK, Peterborough, United Kingdom, pp. 117-122.* URL: https://uhra.herts.ac.uk/handle/2299/21780?show=full. Accessed on 05.05.2022

Fitt, B.D.L., Brun, H., Barbetti, M.J., and Rimmer, S.R. 2006. World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on Oilseed Rape (*Brassica napus*). *Eur. J. Plant. Pathol.* 114: 3–15. doi:10.1007/s10658-005-2233-5.

Flor, H.H. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol. 9*: 275–296. doi:10.1146/annurev.py.09.090171.001423.

FNR 2022. Biodiesel. URL: https://biokraftstoffe.fnr.de/kraftstoffe/biodiesel. Accessed on 05.03.2022

Friedt, W., Tu, J., and Fu, T. 2018. Academic and economic importance of *Brassica napus* rapeseed. In: Liu, S., Snowdon, R., and Chalhoub, B. eds. The *Brassica napus* Genome. Springer International Publishing, Cham. 1–20. doi:10.1007/978-3-319-43694-4_1.

Frieß, J.L., Breckling, B., Pascher, K., and Schröder, W. 2020. Case study 2: oilseed rape (*Brassica napus* L.). In: A. von Gleich and W. Schröder, eds. Gene drives at tipping points. Springer International Publishing, Cham. 103–145. doi:10.1007/978-3-030-38934-5_5.

Gladders , P , Dyer , C , Fitt , B D L , Evans , N , van den Bosch , F , Baierl , A , Turner , J , Walters , K , Northing , P , Sutherland , K G , Oxley , S J P , Foster , V , Ellerton , D , Myers , N , Selley , A , Ashworth , M & Hall , B. 2005 , Pest and disease management system for supporting winter oilseed rape decisions (PASSWORD) – validation phase. HGCA Project Report , no. 390 , HGCA.

Grasso, V., Palermo, S., Sierotzki, H., Garibaldi, A., and Gisi, U. 2006. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest. Manag. Sci.* 62: 465–472. doi:10.1002/ps.1236.

Guo, X.W., Fernando, W.G.D., and Entz, M. 2005. Effects of crop rotation and tillage on blackleg disease of canola. *Can. J. Plant Pathol.* 27: 53–57. doi:10.1080/07060660509507193.

Hammond, K.E., and Lewis, B.G. 1987. Differential responses of oilseed rape leaves to *Leptosphaeria maculans. Trans. Brit. Mycol. Soc.* 88: 329–333. doi:10.1016/S0007-1536(87)80006-2.

Huang, Y.-J., Mitrousia, G.K., Sidique, S.N.M., Qi, A., and Fitt, B.D.L. 2018. Combining *R* gene and quantitative resistance increases effectiveness of cultivar resistance against *Leptosphaeria maculans* in *Brassica napus* in different environments. *PLoS ONE* 13: e0197752. doi:10.1371/journal.pone.0197752.

Hwang, S.-F., Strelkov, S., Peng, G., Ahmed, H., Zhou, Q., and Turnbull, G. 2016. Blackleg (*Leptosphaeria maculans*) severity and yield loss in canola in Alberta, Canada. *Plants* 5: 31. doi:10.3390/plants5030031.

Jørgensen, L.N., and Heick, T.M. 2021. Azole use in agriculture, horticulture, and wood preservation – Is it indispensable? *Front. Cell. Infect. Microbiol.* 11: 730297. doi:10.3389/fcimb.2021.730297.

Joseph-Horne, T., and Hollomon, D.W. 2006. Molecular mechanisms of azole resistance in fungi. *FEMS Microbiol. Lett.* 149: 141–149. doi:10.1111/j.1574-6968.1997.tb10321.x.

Kachlicki, P., and Jetail Jedryczka , M. 1994. Properties of *Phoma lingam* (Tode ex Fr.) Desm. isolates from Poland II. Secondary metabolites production. *Phytopathologia Polonica*,19: 81–86. ISSN: 1230-0462

Kaczmarek, J., and Jedryczka, M. 2011. Characterization of two coexisting pathogen populations of *Leptosphaeria* spp., the cause of stem canker of *brassicas*. *Acta Agrobot.* 64. doi:DOI: https://doi.org/10.5586/aa.2011.012.

Liu, Z., Latunde-Dada, A.O., Hall, A.M., and Fitt, B.D.L. 2014. Phoma stem canker disease on oilseed rape (*Brassica napus*) in China is caused by *Leptosphaeria biglobosa* '*brassicae*'. *Eur. J. Plant Pathol.* 140: 841–857. doi:10.1007/s10658-014-0513-7.

Lô-Pelzer, E., Bousset, L., Jeuffroy, M.H., Salam, M.U., Pinochet, X., Boillot, M., and Aubertot, J.N. 2010. SIPPOM-WOSR: A simulator for integrated pathogen population management of phoma stem canker on winter oilseed rape. *Field Crops Res.* 118: 73–81. doi:10.1016/j.fcr.2010.04.007.

McDonald, B.A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* 40: 349–379. doi:10.1146/annurev.phyto.40.120501.101443.

Mendes-Pereira, E., Balesdent, M. H., Brun, H., and Rouxel, T. 2003. Molecular phylogeny of the *Leptosphaeria maculans- L. biglobosa* species complex. *Mycol. Res.* 107: 1287–1304. doi:10.1017/S0953756203008554.

Nagaharu, U. 1935. Genome analysis in Brassica with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japan. J. Bot.* 7: 389–452.

Naseri, B., Davidson, J.A., and Scott, E.S. 2008. Survival of *Leptosphaeria maculans* and associated mycobiota on oilseed rape stubble buried in soil. *Plant Pathol.* 57: 280–289. doi:10.1111/j.1365-3059.2007.01768.x.

OECD 1997. Consensus document on the biology of *Brassica napus* L. (Oilseed Rape). Organization for economic cooperation and development (OECD), Paris. URL: https://www.oecd.org/science/biotrack/27531440.pdf. Accessed on 05. 03. 2022.

Ogle, H.J., and Dale, M. 1997. Disease management: cultural practices. In: Brown, J.F., and Ogle, H.J., (eds.) Plant pathogens and plant diseases. Rockvale Publications, Armidale, NSW, Australia, 390-404.

Olivier, C. 2012. 2012 Aster yellow outbreak in Saskatchewan. Agriculture and Agri-Food Canada Research Centre, Saskatoon, Canada. URL: https://www1.agric.gov.ab.ca/$Department/deptdocs.nsf/all/crop14324/$FILE/au-2013-olivier-aster-yellows.pdf. Accessed on: 05.04.2022

Orlovius, K. 2003. Fertilizing for high yield and quality oilseed rape. International Potash Institute, Basel, Switzerland. URL: https://www.ipipotash.org/uploads/udocs/No%2016%20Oilseed%20rape.pdf. Accessed on: 02. 04. 2022.

Peng, G., Fernando, W.G.D., Kirkham, C.L., Lange, R., Kutcher, R.H., McLaren, D., Johnson, E., and Turkington, K. 2012. Mitigating the risk of blackleg disease of canola using fungicide strategies. Saskatoon, Canada. URL: https://harvest.usask.ca/bitstream/handle/10388/9101/G.%20Peng%20et%20al.%2c%202012b.pdf?sequence=1&isAllowed=y. Accessed on: 05.02.2022.

Peng, G., Liu, X., McLaren, D.L., McGregor, L., and Yu, F. 2020. Seed treatment with the fungicide fluopyram limits cotyledon infection by *Leptosphaeria maculans* and reduces blackleg of canola. *Can. J. Plant Pathol.* 42: 480–492. doi:10.1080/07060661.2020.1725132.

Pilet, M.L., Delourme, R., Foisset, N., and Renard, M. 1998. Identification of QTL involved in field resistance to light leaf spot (*Pyrenopeziza brassicae*) and blackleg resistance (*Leptosphaeria maculans*) in winter rapeseed (*Brassica napus* L.): Theor. Appl. Genet. 97: 398–406. doi:10.1007/s001220050909.

Poland, J.A., Balint-Kurti, P.J., Wisser, R.J., Pratt, R.C., and Nelson, R.J. 2009. Shades of gray: the world of quantitative disease resistance. *Trends in Plant Sci.* 14: 21–29. doi:10.1016/j.tplants.2008.10.006.

Raboanatahiry, N., Li, H., Yu, L., and Li, M. 2021. Rapeseed (*Brassica napus*): Processing, utilization, and genetic improvement. *Agronomy* 11: 1776. doi:10.3390/agronomy11091776.

Raman, H., Raman, R., Diffey, S., Qiu, Y., McVittie, B., Barbulescu, D.M., Salisbury, P.A., Marcroft, S., and Delourme, R. 2018. Stable quantitative resistance loci to blackleg disease in canola (*Brassica napus* L.) over continents. *Front. Plant Sci.* 9: 1622. doi:10.3389/fpls.2018.01622.

Rouxel, T., and Balesdent, M.H. 2005. The stem canker (blackleg) fungus, *Leptosphaeria maculans* , enters the genomic era. *Mol. Plant Pathol.* 6: 225–241. doi:10.1111/j.1364-3703.2005.00282.x.

Shah, U.A., Kotta-Loizou, I., Fitt, B.D.L., and Coutts, R.H.A. 2020. Mycovirus-induced hypervirulence of *Leptosphaeria biglobosa* enhances systemic acquired resistance to *Leptosphaeria maculans* in *Brassica napus*. *MPMI* 33: 98–107. doi:10.1094/MPMI-09-19-0254-R.

Sierotzki, H., and Scalliet, G. 2013. A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathol.* 103: 880–887. doi:10.1094/PHYTO-01-13-0009-RVW.

Snowdon, R., Lühs, W., and Friedt, W. 2007. Oilseed rape. In: Kole, C., ed. Oilseeds. Springer, Berlin- Heidelberg, Germany. 55–114. doi:10.1007/978-3-540-34388-2_2.

Söchting, H.P., and Verreet, J.-A. 2004. Effects of different cultivation systems (soil management, nitrogen fertilization) on the epidemics of fungal diseases in oilseed rape (*Brassica napus* L. *var. napus*). *J. Plant Dis. Prot.* 111: 1–29. doi:10.1007/BF03356129.

Sprague, S.J., Balesdent, M.-H., Brun, H., Hayden, H.L., Marcroft, S.J., Pinochet, X., Rouxel, T., and Howlett, B.J. 2006. Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. *Eur. J. Plant Pathol.* 114: 33–40. doi:10.1007/s10658-005-3683-5.

Sprague, S.J., Marcroft, S.J., Lindbeck, K.D., Ware, A.H., Khangura, R.K., and Van de Wouw, A.P. 2018. Detection, prevalence and severity of upper canopy infection on mature *Brassica napus* plants caused by *Leptosphaeria maculans* in Australia. *Crop Pasture Sci.* 69: 65. doi:10.1071/CP17140.

Statista 2022. Leading producing countries of rapeseed in 2020/2021. URL: https://www.statista.com/statistics/263930/worldwide-production-of-rapeseed-by-country/. Accessed on: 05.04. 2022.

Steed, J.M., Baierl, A., and Fitt, B.D.L. 2007. Relating plant and pathogen development to optimise fungicide control of phoma stem canker (*Leptosphaeria maculans*) on winter

oilseed rape (*Brassica napus*). Eur. J. Plant Pathol. 118: 359–373. doi:10.1007/s10658-007-9137-5.

Stonard, J.F., Latunde-Dada, A.O., Huang, Y.-J., West, J.S., Evans, N., and Fitt, B.D.L. 2010. Geographic variation in severity of phoma stem canker and *Leptosphaeria maculans*/*L. biglobosa* populations on UK winter oilseed rape (*Brassica napus*). Eur. J. Plant Pathol. 126: 97–109. doi:10.1007/s10658-009-9525-0.

Van de Wouw, A.P., Scanlan, J.L., Marcroft, S.J., Smith, A.J., Sheedy, E.M., Perndt, N.W., Harrison, C.E., Forsyth, L.M., and Idnurm, A. 2021. Fungicide sensitivity and resistance in the blackleg fungus, *Leptosphaeria maculans*, across canola growing regions in Australia. *Crop Pasture Sci.* 72: 994. doi:10.1071/CP21369.

West, J.S., Fitt, B.D.L., Leech, P.K., Biddulph, J.E., Huang, Y.-J., and Balesdent, M.-H. 2002. Effects of timing of *Leptosphaeria maculans* ascospore release and fungicide regime on phoma leaf spot and phoma stem canker development on winter oilseed rape (*Brassica napus*) in southern England: *L. maculans* spore release and disease development. *Plant Pathol.* 51: 454–463. doi:10.1046/j.1365-3059.2002.00726.x.

Williams, R. H., and Fitt B. D. L. 1999. Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. *Plant Pathol.* 48: 161–175. doi:10.1046/j.1365-3059.1999.00333.x.

Zheng, X., Koopmann, B., Ulber, B., and von Tiedemann, A. 2020. A global survey on diseases and pests in oilseed rape- current challenges and innovative strategies of control. *Front. Agron.* 2: 590908. doi:10.3389/fagro.2020.590908.

Zinke, O. 2019. Europäischer Rapsanbau steckt in der Krise. URL: https://www.agrarheute.com/markt/marktfruechte/europaeischer-rapsanbau-steckt-krise-560717. Accessed on: 02. 04. 2022.

*pathogens*

MDPI

*Article*

# Efficacy of Blackleg Major Resistance Genes in *B. napus* in Germany

**Dima Alnajar *, Andreas von Tiedemann and Birger Koopmann**

Plant Pathology and Crop Protection, University of Goettingen, 37077 Goettingen, Germany; atiedem@gwdg.de (A.v.T.); bkoopma@gwdg.de (B.K.)
* Correspondence: dima.alnajar@agr.uni-goettingen.de

**Abstract:** *Leptosphaeria maculans* is one of the major pathogens of oilseed rape (*B. napus*). It causes blackleg disease, which accounts for significant yield losses worldwide. Using cultivars that harbor major resistance (*R*) genes is one of the most effective control methods. However, the efficacy of major *R* genes is related to the frequency of the corresponding avirulence (*Avr*) genes in a *L. maculans* population. In this paper, we report the *Avr* profiles of *L. maculans* populations and the ratio of its mating types in Northern and Central regions of Germany. Eleven *Avr* genes in five-hundred and seventy-four isolates were characterized either by applying cotyledon tests on a *B. napus* differential set or by amplifying avirulence gene-specific PCR markers. Fifty-two races were determined, among which the most dominant race was *Avrlm6, -7, -11, AvrlepR1, -R2*. Results showed that the resistance gene *Rlm2* is 100% ineffective, some other major *R* genes such as *Rlm1, Rlm3, Rlm4* and *LepR3* are partially effective (with corresponding *Avr* frequencies $\leq$ 42%), while *LepR1, LepR2, Rlm6, Rlm11* and *Rlm7* can still provide relatively effective resistance in the German fields investigated (with corresponding *Avr* frequencies of 63–100%). Sexual reproduction is a factor that enhances the potential of *L. maculans* to evolve under selection pressure. Mating types of the *L. maculans* populations did not deviate from the ratio of 1:1 in the examined regions, indicating that sexual reproduction and ascospores play central roles in the *L. maculans* lifecycle. Overall, this study provides an important dataset for the establishment of a strategic plan to preserve the efficacies of major *R* genes in Germany by applying cultivar rotations of oilseed rape.

**Keywords:** *L. maculans*; *L. biglobosa*; *B. napus*; oilseed rape; blackleg disease; phoma stem canker; qualitative resistance; major resistance genes



**Citation:** Alnajar, D.; von Tiedemann, A.; Koopmann, B. Efficacy of Blackleg Major Resistance Genes in *B. napus* in Germany. *Pathogens* **2022**, *11*, 461. https://doi.org/10.3390/pathogens11040461

Academic Editor: Donato Gerin

Received: 17 February 2022
Accepted: 10 April 2022
Published: 12 April 2022



## 1. Introduction

Blackleg disease (phoma stem canker) is an economically important disease in many oilseed rape-growing areas in the world [1]. The causal agent of the disease is a two-species complex: *Leptosphaeria maculans* (anamorph = *Plenodomus lingam*) and *Leptosphaeria biglobosa* (anamorph = *Plenodomus biglobosus*) [2,3]. Both species coexist in the field. However, the ratio of *L. maculans* to *L. biglobosa* in a region is decisive for disease severity, so that *L. maculans* is mainly responsible for significant yield losses [4]. *L. maculans* was reported to be dominant in Germany and other western European countries [1].

Having a complex lifecycle with two reproduction systems and different dispersal mechanisms, *L. maculans* has a high evolutionary potential that allows it to readily adapt to new conditions, such as introducing new *R* genes in its host plant [5]. Generally, the fungus survives saprophytically on stubbles of a previous season by means of the sexual fruiting bodies, pseudothecia. Once mature, pseudothecia eject wind-borne ascospores, resulting in a primary infection of host plants. Ascospores germinate on cotyledons and young leaves. Hyphae grow biotrophically to form characteristic phoma lesions, mostly with asexual fruiting bodies, pycnidia. The latter contain pycnidiospores, which can be spread by rain splashes. Their spread may finally result in a secondary infection. The fungus grows

symptomless from leaves through petioles to stems. Once in the stem base, the fungus turns necrotrophic and causes canker [1,6]. Additionally, *L. maculans* can be seed-transmitted [7]. Studies describing a high diversity in *L. maculans* populations have repeatedly emphasized the importance of sexual reproduction for generating wide genetic variation [8,9]. However, despite its importance, sexual reproduction might not be dominant in some seasons. Such cases were reported in western Canada, where ascospores were not detected in the air in some years, although infection was severe. Thus, it was concluded that ascospores did not form the main inoculum. Instead, pycnidiospores represented the alternative main source of inoculum [10]. *L. maculans* has two mating types, *MAT1-1* and *MAT1-2* [11]. According to the random mating hypothesis, the mating type frequencies in a population should be 1:1 under random sexual outcrossing [12]. Defining the idiomorph ratios in a *L. maculans* population helps to determine the source of the primary inoculum, and thus the speed expected for the pathogen to evolve new races in a specific region.

Integrated management of phoma stem canker includes stubble management, crop rotation, applying fungicides and using resistant cultivars [13,14]. Two types of resistance are known: polygenic, non-race specific, quantitative resistance and major gene, race-specific, qualitative resistance [1,15]. The immune system in plants is described by a "Zigzag" model of several phases [16]. Once *L. maculans* interacts with the host, the fungus initially confronts extracellular pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs), and this recognition results in PAMP-triggered immunity (PTI). The pathogen excretes specific effectors to suppress PTI. If the plant cannot recognize these effectors, an effector-triggered susceptibility (ETS) will be initiated. Otherwise, a specific recognition of effectors will activate an effector-triggered immunity (ETI) [16]. Effector-specific recognition happens according to the gene-for-gene concept, in which each *Avr* gene in the pathogen has a counterpart major *R* gene in the host [17,18]. The classic perception of the recognition process depicts it as a direct interaction between a plant receptor protein and a pathogen avirulence protein [18,19]. However, a more contemporary point of view speculates that *R* gene(s) in a plant monitor the occurrence of modulations of host cell components that are targeted by the pathogen to prepare the cell environment for the invasion [20,21]. Through the co-evolution of pathogens and their hosts, natural selection allows a pathogen population to modify their *Avr* profile to generate effectors able to successfully suppress PTI [16].

New emerged isolates have previously been categorized into pathogenicity groups, including *L. biglobosa,* until Shoemaker and Brun [2] provided the final taxonomic evidence to separate *L. biglobosa* as a distinct species. Since then, there has been a considerable gain of knowledge about major blackleg resistance genes, which have also been incorporated into actual tester sets, allowing the definition of races (up to $2^n$). Characterization of *L. maculans* by their races was suggested by Balesdent et al. [22]. This characterization relies on differential cotyledon reactions of tester lines to individual isolates of *L. maculans* based on *Avr–R* gene interactions. *Avr–R* gene interactions in the *L. maculans-B. napus* pathosystem can be more complex than involving two genes. On the one hand, a redundant recognition of a single *Avr* gene can be displayed by two major *R* genes [23]. Larkan et al. demonstrated that the avirulence gene *Avrlm1* encodes effectors that can trigger the two major *R* genes *LepR3* and *Rlm1* [24]. On the other hand, there are cases where two *Avr* genes must act together to be able to trigger one *R* gene, the so-called two-gene-for-one-gene interaction. For example, *Rlm10*-mediated recognition can be triggered only if both *Avrlm10A* and *Avrlm10B* are present together [25]. Additionally, it was reported that the functional allele *Avrlm7* masks the recognition of *Avrlm9* and *Avrlm3* due to an epistatic interaction effect [26,27]. In *L. maculans*, fourteen *Avr* genes have been identified so far. Eight of them were cloned: *Avrlm1-L3* [28], *Avrlm2* [29], *Avrlm3* [26], *Avrlm4-7* [30], *Avrlm5-9* [27,31], *Avrlm6* [32], *Avrlm10* [25] and *Avrlm11* [33]. On the side of the host, in *B. napus*, an even larger number of corresponding major *R* genes were described, such as *Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, Rlm10, Rlm11, RlmS (BLMR1.2),*

*Rlm13, LepR1, LepR2, LepR3 (BLMR1.1)* and *LepR4*. Only three of them, *Rlm2, Rlm9* and *LepR3,* were cloned [24,34–40].

Sowing a specific commercial cultivar harboring a major *R* gene in a region over years results in high natural selection pressure. Hence, new *L. maculans* races evolve that can overcome the introduced major *R* gene. This is called a "boom and bust" cycle [5]. The amplitude of a "boom and bust" cycle of a major *R* gene differs among fungal phytopathogens. For *L. maculans*, several studies have documented the potential lifespan of a major *R* gene when intensively deployed under experimental conditions or at a commercial level. For example, Brun et al. reported that *Rlm6* turned ineffective after three growing seasons in field experiments [41]. On a commercial level, breakdowns of major *R* genes have been observed within three to five years after their introduction into the market in many oilseed rape-growing countries, such as the efficacy loss of *Rlm1* in France, *Rlm3* in western Canada and "sylvestris"-derived resistance, namely *Rlm1* and *LepR3*, in Australia [42–44].

Setting a strategic regional plan to rotate cultivars harboring major *R* genes is essential to expand the efficacy longevity of major *R* genes. The reason is that using qualitative resistance is only reasonable as long as the corresponding *Avr* gene is dominant in the population. However, a reliable plan for major *R* gene rotation requires regular updating of the *Avr* profile of regional *L. maculans* populations [45]. Monitoring of *Avr* gene frequencies is thus crucial for practical recommendations for farmers and breeders.

The last study that investigated *Avr* gene frequencies in *L. maculans* populations in Germany was based on samples collected in the growing seasons of 2011 and 2012 [46]. Here, we aimed not only to update the *Avr* profile of *L. maculans* populations, but also to expand the range of the tested *Avr* genes by including *Avrlm6, Avrlm11, AvrlepR1, AvrlepR2* and *AvrlepR3,* which have not been investigated in Germany so far. In addition, special attention was given to the change in *Avrlm7* frequency, since *Rlm7* has been known as the most effective commercialized major *R* gene in Germany in recent years. Additionally, this study aimed to check whether *L. maculans* population mating types deviate from the hypothesized 1:1 ratio in northern and central Germany.

## 2. Results

Six hundred isolates were collected during three seasons from 2017 to 2020 from seven regions in four provinces in northern and central Germany (Figure 1). Using the primers ITS5/ITS4 targeting the internal transcribed spacer (ITS) regions, 26 isolates were identified as *L. biglobosa* while 574 isolates were assigned to *L. maculans.* In the pathogenicity test, the 26 *L. biglobosa* isolates caused only very small necrotic leaf spots with no pycnidia on the cotyledons. Additionally, in the susceptible check, cultivar Westar did not show more severe infection when inoculated with the *L. biglobosa* isolates. The 574 *L. maculans* isolates were further characterized to determine their races and their mating types.

### *2.1. Efficacy of Major Resistance Genes in German Fields*

The 574 isolates assigned to *L. maculans* were used to monitor the *Avr* profile of the pathogen population and thus the *R* gene efficacies. Eleven avirulence genes were characterized (*Avrlm1, Avrlm2, Avrlm3, Avrlm4, Avrlm6, Avrlm7, Avrlm9, Avrlm11, AvrlepR1, AvrlepR2* and *AvrlepR3*), either phenotypically by performing cotyledon tests on an oilseed rape differential set or genotypically by using *Avr* gene-specific PCR primers.

Results of the pathogenicity test provide evidence that all the tested isolates were virulent on differential lines that harbored *Rlm2* and *Rlm9* (Figure 2). This shows that *Rlm2* is ineffective in the explored regions. However, the 100% virulence frequencies on the *Rlm9* differentials were expected since the trap variety NK Bravour harbors *Rlm9*. In contrast, Figure 2 showed that *AvrlepR1* proved to be the most abundant avirulence gene with 100% presence in all investigated regions, except in Peine, where two isolates virulent on the differential line Topas-*LepR1* were detected, representing 2% of Peine's population and 0.3% of the whole isolate collection.

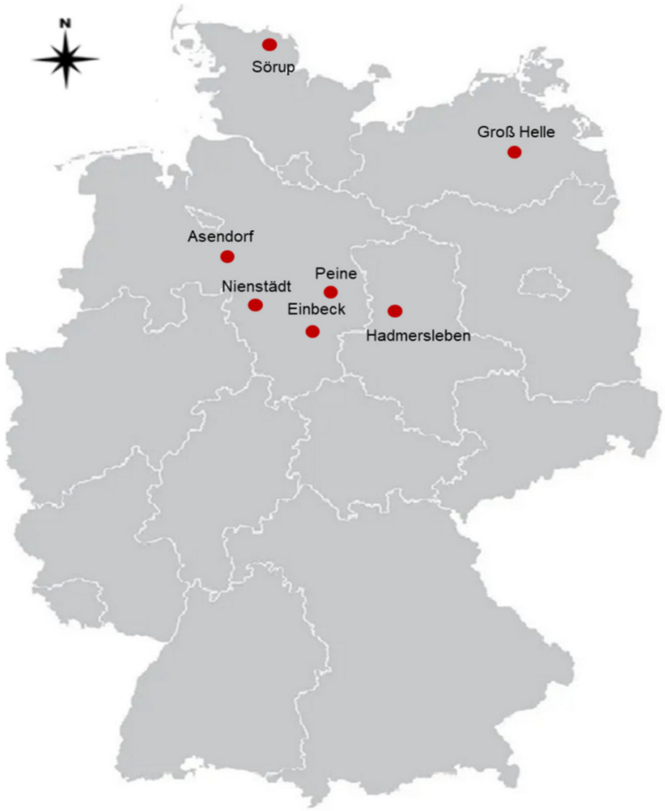



**Figure 1.** Field trial locations in central and northern Germany from which leaf samples with phoma lesions were collected to race type *L. maculans* populations (2017 to 2020).

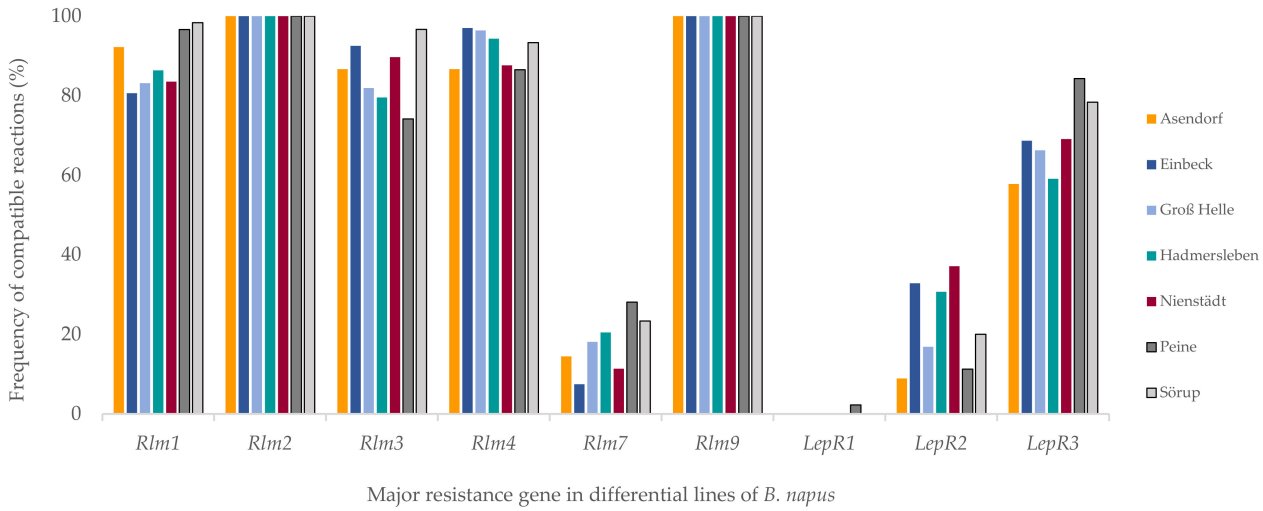



**Figure 2.** Virulence frequencies of *L. maculans* isolates originating from different fields tested on a *B. napus* differential set. Cotyledon tests were conducted with 574 isolates. Field sites and numbers of tested isolates: Hadmersleben, n = 88; Groß Helle, n = 83; Nienstädt, n = 97; Einbeck, n = 67; Sorüp = 60; Asendorf = 90, Peine, n = 89. Isolates were collected from 2017 to 2020.

There was a drastic increase in the frequency of *avrlm7* isolates (i.e., isolates virulent on *Rlm7*) demonstrated in this study when compared with the results of our previous work on *Rlm7* in Germany (2011–2012) [46]. A comparative analysis of both studies showed that the *avrlm7* isolate's frequency increased within 5–7 years from 0.9% to 17.6% on average in all investigated regions. This study confirmed that *Rlm7* has become less effective in Germany since 2011–2012, only a few years after its commercial release. According to Figure 2, the percentage of *avrlm7* isolates ranged from 7% in Einbeck to 28% in Peine.

Frequencies of compatible interactions on *Rlm1, Rlm3, Rlm4* and *LepR3* varied from region to region. Phenotypical data revealed that the functional *Avrlm1* ranged from 2% to 19%, *Avrlm3* from 3% to 26%, *Avrlm4* from 3% to 13% and *LepR3* from 16% to 42%. These results indicate that the referred *R* genes are still partially effective. Interestingly, although the alternating functionality supposed due to the masking effect between *Avrlm7* and *Avrlm3* can be recognized in Figure 2, 17 *avrlm7* isolates out of 101 were virulent on the differential Topas-*Rlm3,* despite the absence of the functional *Avrlm7*.

*LepR2* is also partially effective. However, with a frequency of functional *AvrlepR2* ranging from 63% to 91%, *LepR2* can be considered similarly effective as *Rlm7* in some regions, and thus, the two can be used in rotation with each other to avoid or delay the further resistance breakdown of both. The results in Figure 2 emphasize the importance of considering the regional differences for *L. maculans* management strategies. For example, Figure 2 shows that, while *LepR2* is more effective than *Rlm7* in Peine, the opposite is true in Einbeck and Nienstädt.

Since we did not have access to *B. napus* lines harboring the major *R* genes *Rlm6* and *Rlm11*, we used specific PCR primers to test for *Avrlm6* and *Avrlm11*. To our knowledge, there are no reports from other research groups who have differential lines including *Rlm6* and *Rlm11* about masking effects of *Avr* genes that hinder the recognition of *Avrlm6* or *Avrlm11.* Additionally, it was reported that deletion is the major mechanism of gaining virulence in these *Avr* genes [33,47]. Therefore, we assumed that the results of the PCR avirulence gene tests would likely match the results of the phenotyping by cotyledon tests. Figure 3 shows that the frequency of *Avrlm6* ranged from 88% to 100%, while the frequency of *Avrlm11* was 72% to 95%. Consequently, it can be concluded that *Rlm6* and *Rlm11* are relatively effective, and their efficacies are comparable to that of *Rlm7*. However, it is noteworthy that there are no reports stating the introduction of these two genes into commercial cultivars in Germany.

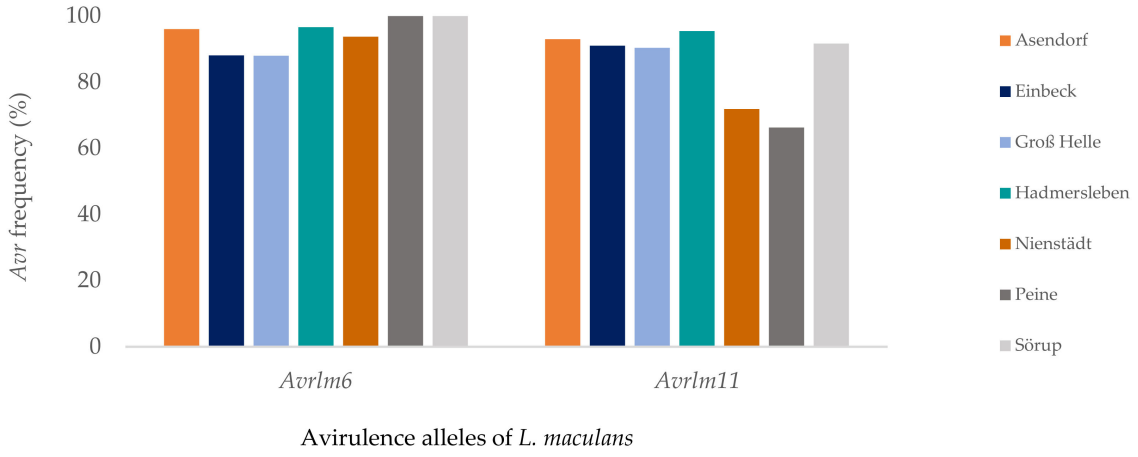



**Figure 3.** Frequencies of avirulence genes *Avrlm6* and *Avrlm11* in *L. maculans* isolates collected from different fields in Germany tested by PCR. In total, 574 isolates were tested. Field sites and numbers of tested isolates: Hadmersleben, n = 88; Groß Helle, n = 83; Nienstädt, n = 97; Einbeck, n = 67; Sorüp = 60; Asendorf = 90, Peine, n = 89.

### *2.2. L. maculans Races in German Populations*

In general, 52 races were described among the 574 *L. maculans* isolates collected from 7 sites in Germany. However, richness in races according to the Margalef index differed between regions. Table 1 illustrates that Nienstädt showed the highest race diversity with 25 races and a Margalef index of 5.25, whereas Sörup displayed the lowest race diversity with 14 races and a Margalef index of 3.18.

**Table 1.** Number of *L. maculans* and *L. biglobosa* isolates and Margalef index indicating the local population diversity of *L. maculans* races at the different sites.

| Site | Province | No. of Isolates | *L. biglobosa* | No. of Races | Margalef Index |
|---|---|---|---|---|---|
| Nienstädt | Lower Saxony | 99 | 2 | 25 | 5.25 |
| Groß Helle | Mecklenburg-Western Pomerania | 83 | 0 | 21 | 4.53 |
| Peine | Lower Saxony | 97 | 8 | 20 | 4.23 |
| Einbeck | Lower Saxony | 71 | 4 | 16 | 3.57 |
| Asendorf | Lower Saxony | 100 | 10 | 18 | 3.56 |
| Hadmersleben | Saxony Anhalt | 90 | 2 | 16 | 3.35 |
| Sörup | Schleswig-Holstein | 60 | 0 | 14 | 3.18 |

The races presented in Table 2 are based on the phenotyping tests and the PCR tests of *Avrlm6* and *Avrlm11*. Since *LepR3* is assumed to interact with *Avrlm1*, it was not possible to ensure the presence of *AvrlepR3* distinctly from *Avrlm1* in isolates avirulent on both *LepR3* and *Rlm1*. Therefore, these isolates were marked with asterisks to draw attention to the possible redundance. Some researchers hypothesize that *Avrlm1* and *AvrlepR3* are identical, and that *AvrlepR3* is a hypothetical gene [48]. However, there were races in our tested collection that were virulent on *Rlm1* and avirulent on *LepR3*. This indicates that this assumption may not be true.

**Table 2.** Race spectrum of *L. maculans* populations from seven field sites in Germany collected from 2017 to 2020. Races are described based on phenotypic characterization of *Avrlm1, Avrlm2, Avrlm3, Avrlm4, Avrlm7*, *LepR1, LepR2* and *LepR3. Avrlm6* and *Avrlm11* were characterized based on specific PCR primers.

| *L. maculans* Races | Percentage of Total Number of Isolates Collected Per Region | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Asendorf | Einbeck | Groß Helle | Hadmersleben | Peine | Sörup | Nienstädt | Total |
| *Avrlm6, -7, -11, AvrlepR1, -R2* | 31 | 25 | 40 | 28 | 29 | 43 | 16 | 30.8 |
| *Avrlm6, -7, -11, AvrlepR1, -R2, -R3* | 21 | 7 | 10 | 17 | 4 | 10 | 9 | 11.8 |
| *Avrlm6, -7, -11, AvrlepR1* | 5 | 15 | 7 | 19 | 4 | 17 | 13 | 11.5 |
| *Avrlm3, -6, -11, AvrlepR1, -R2* | 5 | 7 | 10 | 4 | 12 | 2 | 6 | 7.1 |
| *Avrlm1, -6, -7, -11, AvrlepR1, -R2, -R3* * | 4 | 11 | 4 | 1 | 0 | 2 | 4 | 3.7 |
| *Avrlm6, -7, AvrlepR1, -R2* | 0 | 0 | 2 | 0 | 9 | 0 | 7 | 3.1 |
| *Avrlm4, -6, -7, -11, AvrlepR1, -R2, -R3* | 4 | 0 | 1 | 3 | 3 | 2 | 3 | 2.6 |
| *Avrlm4, -6, -7, -11, AvrlepR1, -R2* | 6 | 0 | 0 | 0 | 3 | 0 | 3 | 2.1 |
| *Avrlm6, -7, AvrlepR1* | 1 | 1 | 0 | 0 | 2 | 2 | 7 | 2.1 |
| *Avrlm3, -6, -11, AvrlepR1, -R2, -R3* | 3 | 0 | 2 | 3 | 1 | 0 | 1 | 1.7 |
| *Avrlm6, -11, AvrlepR1, -R2* | 2 | 0 | 0 | 0 | 1 | 10 | 0 | 1.6 |
| *Avrlm7, AvrlepR1, -R2* | 0 | 6 | 0 | 0 | 0 | 0 | 5 | 1.6 |
| *Avrlm1, -6, -7, -11, AvrlepR1, -R3* * | 0 | 0 | 1 | 3 | 0 | 0 | 4 | 1.4 |
| *Avrlm1, -6, -7, AvrlepR1, -R2, -R3* * | 0 | 0 | 5 | 1 | 2 | 0 | 1 | 1.4 |
| *Avrlm3, -6, AvrlepR1, -R2* | 0 | 0 | 0 | 0 | 7 | 0 | 1 | 1.4 |
| *Avrlm3, -6, -11, AvrlepR1* | 0 | 0 | 1 | 6 | 1 | 0 | 1 | 1.4 |
| *Avrlm7, -11, AvrlepR1, -R2* | 1 | 0 | 5 | 1 | 0 | 0 | 1 | 1.2 |
| *Avrlm1, -3, -6, -11, AvrlepR1, -R2, -R3* * | 0 | 0 | 1 | 6 | 0 | 0 | 0 | 1.0 |
| *Avrlm1, -4, -6, -7, -11, AvrlepR1, -R2, -R3* * | 1 | 1 | 1 | 0 | 1 | 0 | 2 | 1.0 |

**Table 2.** *Cont.*

| *L. maculans* Races | Percentage of Total Number of Isolates Collected Per Region | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Asendorf | Einbeck | Groß Helle | Hadmersleben | Peine | Sörup | Nienstädt | Total |
| *Avrlm4, -6, -7, AvrlepR1, -R2* | 0 | 0 | 0 | 0 | 4 | 0 | 2 | 1.0 |
| *Avrlm6, -7, -11, AvrlepR1, -R3* | 2 | 4 | 0 | 1 | 0 | 0 | 0 | 1.0 |
| *Avrlm3, -6, AvrlepR1, -R2, -R3* | 1 | 0 | 0 | 0 | 1 | 3 | 0 | 0.7 |
| *Avrlm7, AvrlepR1* | 0 | 3 | 0 | 0 | 0 | 0 | 2 | 0.7 |
| *Avrlm7, AvrlepR1, -R2, -R3* | 0 | 3 | 0 | 2 | 0 | 0 | 0 | 0.7 |
| *Avrlm4, -6, -11, AvrlepR1, -R2* | 0 | 0 | 0 | 0 | 1 | 3 | 0 | 0.5 |
| *Avrlm7, AvrlepR1, -R3* | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0.5 |
| *Avrlm7, -11, AvrlepR1* | 0 | 3 | 1 | 0 | 0 | 0 | 0 | 0.5 |
| *Avrlm1, -3, -6, -11, AvrlepR1, -R3* * | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0.3 |
| *Avrlm1, -4, -6, -7, -11, AvrlepR1, -R3* * | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0.3 |
| *Avrlm1, -6, -7, -11, AvrlepR1* | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0.3 |
| *Avrlm1, -6, -7, AvrlepR1, -R3* * | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0.3 |
| *Avrlm3, -6, AvrlepR1* | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0.3 |
| *Avrlm6, -7* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0.3 |
| *Avrlm6, -7, AvrlepR1, -R2, -R3* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0.3 |
| *Avrlm1, -3, -6, AvrlepR1, -R2, -R3* * | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0.2 |
| *Avrlm1, -4, -7, -11, AvrlepR1, -R2, -R3* * | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm1, -4, -7, AvrlepR1, -R2, -R3* * | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm1, -7, -11, AvrlepR1, -R2, -R3* * | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm1, -7, -11, AvrlepR1, -R3* * | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm1, -7, AvrlepR1, -R2, -R3* * | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm1, -7, AvrlepR1, -R3* * | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0.2 |
| *Avrlm3, AvrlepR1, -R2* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm3, -11, AvrlepR1* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm4, -6, -7, -11, AvrlepR1* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm4, -6, -11, AvrlepR1, -R2, -R3* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0.2 |
| *Avrlm4, -6, -7, Avrlm11, AvrlepR1, -R3* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0.2 |
| *Avrlm6, -7, AvrlepR1, -R3* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0.2 |
| *Avrlm6, -11, AvrlepR1, -R2, -R3* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0.2 |
| *Avrlm6, -11, AvrlepR1* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0.2 |
| *Avrlm7, -11, AvrlepR1, -R2, -R3* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0.2 |
| *Avrlm6, AvrlepR1, -R2, -R3* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0.2 |
| *Avrlm3, AvrlepR1, -R2, -R3* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2 |

* Since the major *R* gene in Topas-*LepR3* can be triggered by both *Avrlm1* and *AvrlepR3*, it was not possible to ensure the presence of *AvrlepR3* distinctly from *Avrlm1* in isolates avirulent on both *LepR3* and *Rlm1*. Therefore, such races are marked with an asterisk.

The predominant race in all regions investigated was *Avrlm 6, 7, 11, AvrLepR 1, 2,* which represented 30.8% of the whole races in the tested German *L. maculans* population. The second most dominant race was *Avrlm6, 7, 11, AvrlepR1, 2, 3,* making up 11.8% of the population, and differing from the most dominant one by the presence of *AvrlepR3*. The third dominant race was *Avrlm6, 7, 11, AvrlepR1,* with a share of 11.5%, and the fourth ranking was *Avrlm3, 6, 11, AvrlepR1, 2,* at a rate of 7.1%. The four most dominant races together accounted for 61.3% of all races in the tested German population. An effective oilseed rape cultivar rotation should basically consider these most dominant races.

Isolates of the *L. maculans* population in the explored regions had between two and eight functional avirulence genes (Figure 4). Among them, 43% had five different *Avr* genes, 22% had four *Avr* genes and 20% had six *Avr* genes. Only a few isolates (1%) had two or eight *Avr* genes.

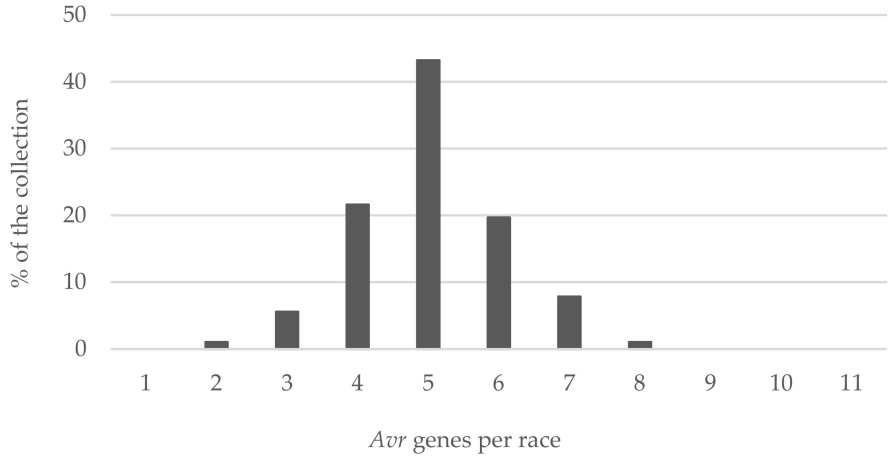



**Figure 4.** Frequencies of *Avr* gene complexity in races of the investigated German *L. maculans* population.

### 2.3. *L. maculans* Mating Type Ratio in German Fields

Mating types were defined using multiplex PCR in 562 isolates (Figure 5). Both idiomorphs of the pathogen existed in each region and Fisher's exact test proved no significant departure from the 1:1 ratio of the two mating types (Table 3). This emphasizes the importance of the annual sexual reproduction in the lifecycle of *L. maculans* in the explored regions. In fact, primary infection in Germany depends on the spread of ascospores, a factor that enhances the ability of the fungus to adapt rapidly to new qualitative major *R* genes implemented in newly commercialized cultivars.

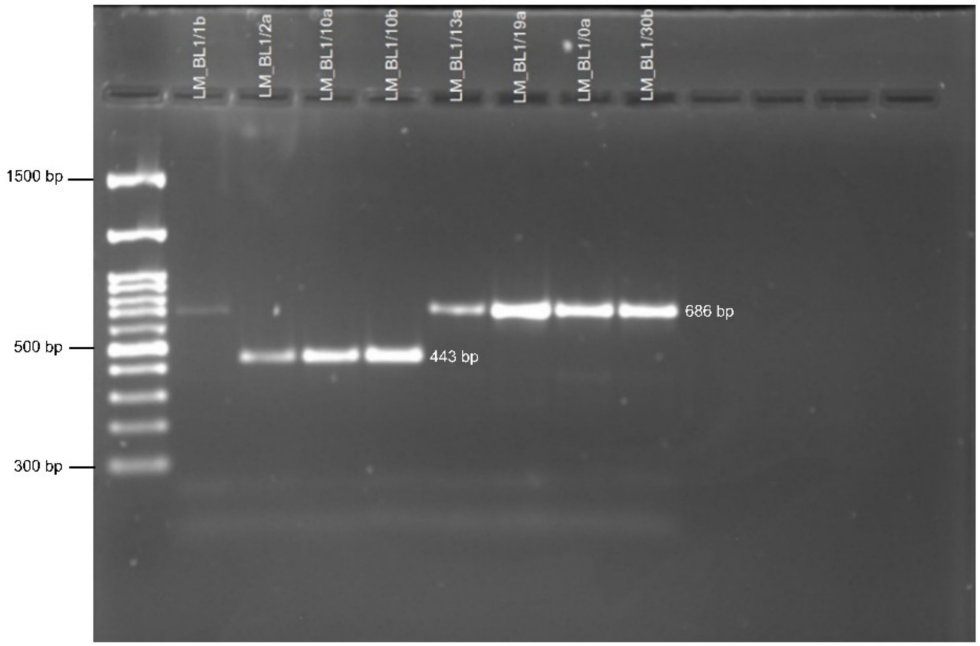



**Figure 5.** Agarose gel electrophoresis of the PCR-amplified mating type loci of *L. maculans* using a multiplex PCR system described by Cozijnsen and Howlett [11]. Three primers were used. The use of a common primer in combination with the *MAT1-1* locus-specific primer results in an amplicon of 686 bp, while the use of it in combination with the *MAT1-2* locus-specific primer affords an amplicon of 443 bp.

**Table 3.** Proportion of mating types of *L. maculans* in an isolate collection from different regions in central and northern Germany. Multiplex PCR was used to define the idiomorphs of the pathogen. Fisher's exact test showed no significant deviation from the 1:1 ratio of the mating types ($p = 0.05$).

| Site | No. of Isolates | *MAT1-1* (%) | *MAT1-2* (%) |
|---|---|---|---|
| Nienstädt | 96 | 47 | 53 |
| Groß Helle | 73 | 44 | 56 |
| Peine | 89 | 42 | 58 |
| Einbeck | 65 | 45 | 55 |
| Asendorf | 94 | 56 | 43 |
| Hadmersleben | 88 | 48 | 52 |
| Sörup | 57 | 37 | 63 |

## 3. Discussion

This is the first study identifying the frequencies of *AvrlepR1*, *AvrlepR2*, *AvrlepR3*, *Avrlm6* and *Avrlm11* in the *L. maculans* population in German fields. Besides, it has updated the *Avr* profiles of *Avrlm1*, *Avrlm2*, *Avrlm3*, *Avrlm4* and *Avrlm7,* which have not been investigated in Germany since 2012 [46]. Cotyledon tests showed that 100% of the isolates were virulent on differential lines harboring *Rlm2*. The absence of *Avrlm2* was also described in northern Germany in isolates sampled in 2011–2012 [46]. Similar results have previously been found in France in 2000–2001 [49]. However, this situation is different on other continents. For example, in western Canada, a survey on samples collected in 2012–2014 showed that *Avrlm2* reached 80% [50]. All our sampled isolates were virulent on the *Rlm9* differential. This can be explained by the use of the *Brassica napus* trap cultivar NK-Bravour that carries the *Rlm9 R* gene. This led to a preselection of *avrlm9*-harboring isolates, whereas *Avrlm9*-harboring isolates were counter-selected.

Epistatic effects, as a mechanism for evading recognition, were reported by *Avrlm7* toward *Avrlm3* [26] and *Avrlm9* [27]. Indeed, the results of our study supported that the presence of the functional *Avrlm7* masked the recognition of *Avrlm3*. Investigations of epistatic mechanisms of *Avrlm7* toward *Avrlm3* and *Avrlm9* revealed that this suppression was caused by neither stopping the expression of *Avrlm3* and *Avrlm9*, nor a physical interaction of the *Avr* effector proteins of *Avrlm3*, *Avrlm9* and *Avrlm4–7* [27,51]. Using protein structure approaches, it was demonstrated that the three effector proteins of these genes belong to a new family of effectors, called *Leptosphaeria* AviRulence-Supressing effectors (LARS). LARS are structurally analogue effectors that differ in their amino acid identities, although they share common targets in the plants [52].

In our study, several compatible interactions on the *Rlm3* differential lines were detected among isolates lacking the functional *Avrlm7.* This indicates that although *Avrlm7* masks the recognition of *Avrlm3*, its absence does not mean that the pathogen has no other means to avoid recognition. A variety of virulence-gain mechanisms in plant pathogens have been described, such as deletion of the *Avr* gene, point mutations that allow the pathogen to avoid recognition despite the presence of the *Avr* gene, amino acid substitution and masking the *Avr* protein through another *Avr* protein [26,47,53]. Gene silencing of *Avrlm3* proved that this *Avr* gene is crucial in *L. maculans* pathogenicity and has an important effect on its lifecycle in *B. napus* [51]. Thus, deletion of the gene can be ruled out by elucidating the virulence of *avrlm7* isolates on *Rlm3*. Plissonneau et al. explained the virulence of *avrlm7* isolates toward *Rlm3* by the high allelic polymorphism of *Avrlm3*, which allows a high level of possible protein isoforms. Therefore, it can be speculated that the alternative mechanism used by the pathogen when the epistatic effect disappears is to substitute an amino acid in the effector protein to allow a so-called "camouflage" type of escaping recognition [51]. Setting strategies for *R* gene management based on restoring the efficacy of *Rlm9* and *Rlm3* by losing the functional *Avrlm7* could mislead farmers into reducing caution in *Rlm7* deployment.

Our results showed that major *R* genes *Rlm1, Rlm3, Rlm4* and *LepR3* are not able to provide sufficient resistance against phoma stem canker in German fields. In the explored

regions, *Avrlm4* isolate frequencies ranged from 3% to 13%. This is quite close to the rate described in a large-scale survey throughout France in 2000–2001 (0–19.5%) [49]. *Avrlm4*-harboring isolates turn virulent when glycine at position 120 in the *Avr* protein is substituted with arginin [54], while *Avrlm1* and *AvrlepR3*, which are suspected to be alleles of the gene *Avrlm1-L3*, turn virulent mainly due to deletion of the whole gene [55,56]. Our results showed that *Avrlm1* frequency ranged from 2% to 19%. A rapid adaptation of the *L. maculans* population toward *Rlm1* was noticed in France, where the rate of *Avrlm1*-harboring isolates made up 83% of the population in 1997–1998 and decreased dramatically to less than 13% in 1999–2000 [42]. Similarly, in Canada, *Avrlm1* frequency did not exceed 5% [45]. In Australia, the efficacy of *Rlm1* resistance in cultivar Surpass 400 notably decreased within three years after commercial release [23], as well as the efficacy of *LepR3*. This is explained by the fact that *Avrlm1* has dual specificity and can trigger both *R* genes: *Rlm1* and *LepR3* [24]. It can be concluded that wherever *Rlm1* efficacy is broken, *LepR3's* lifespan is shorter than that of other *R* genes, in which the corresponding *Avr* gene does not have dual specificity.

We identified a dramatic increase in the frequency of *avrlm7* isolates compared with the 2011–2012 season. The frequency of *avrlm7* isolates increased within five to seven years from 0.9% to 17.6% in fields located in central and northern Germany, and the highest presence of them was in Peine, where the frequency reached 28%. Winter and Koopmann stated that *Rlm7* was the only still effective major *R* gene used in commercial cultivars in Germany [46]. The potential speed of *Rlm7* breakdown was experimentally tested by applying maximum pressure on the *L. maculans* population through sowing cultivars with *Rlm7* for four years, without applying crop rotation or ploughing in the crop debris. Under these experimental conditions, the results revealed that *avrlm7* isolates frequency increased from 0 to 36% within 4 years [54]. In 2006, a study at the European level showed that the *Avrlm7* frequency was 100% in France, Germany, Sweden and Poland [57]. However, the intense deployment of *Rlm7* resulted in a rapid reduction of *Avrlm7* not only in Germany, but also in France. There, *Rlm7* was introduced commercially in 2004. By 2013, the frequency of *Avrlm7* isolates reached an average of 19.5% and a maximum of 45%, depending on the region [58]. In contrast, *Rlm7* is still effective in Canada, with an *Avrlm7* frequency exceeding 98% in 2018–2019 [45]. Hence, the present study calls for a more cautious deployment of *Rlm7*, especially in Europe.

Some major *R* genes in oilseed rape were derived from related *Brassica* species. For instance, while *Rlm11*, *LepR1*, *LepR2* and *LepR3* were introduced into *B. napus* from *B. rapa* [33–35], *Rlm6* originated from *B. juncea* [59]. Our survey provided strong evidence that *LepR1* is the most effective major *R* gene in Germany, with 100% frequency of the isolates harboring *AvrlepR1* in all regions investigated, except in Peine, where 2% of the isolates were virulent on *LepR1*. Although the proportion of the virulent isolates toward *LepR1* was very low, and such isolates were found only in one region, the former observation of the rapid decline in efficacy of *Rlm7* within 5–7 years should prompt to take measures to preserve the efficacy of *LepR1*.

In France, *AvrlepR1*, *AvrlepR2*, *Avrlm6* and *Avrlm11* frequencies in the *L. maculans* population were shown to still be high enough to control the disease in the field [60]. This is in line with our findings in Germany. A significant constraint in the targeted use of *R* genes in the field is a lack of knowledge about their presence in several commercial cultivars registered in Germany. *LepR2*, which is believed to be the same gene or an allelic form of *RlmS*, was reported in cultivars recently registered in France and Germany [61]. Considering the frequency of *avrlepR2* in Germany of 9–37%, as shown in our study, an annual monitoring seems necessary in regions where cultivars harboring *LepR2* are grown. This may help to avoid a situation such as that in Canada, where *AvrlepR1*, *AvrlepR2* and *AvrlepR3* occur already at low frequencies of ca. 19%, 5% and 5–28%, respectively [45].

The major mechanism of virulence gain of *Avrlm6* is a deletion of the gene [47]. Van de Wouw et al. studied isolates collected in Australia over two decades (from 1987 to 2017) and observed fluctuating frequencies of *Avrlm6* from 21% to 80% depending on the year. This behavior appeared to be independent from the commercial introduction of *Rlm6* in

Australia in 2010 [62]. Hence, the study speculated that *Avrlm6* frequency might also be influenced by the intensive deployment of other major *R* genes in Australia [62]. In France, *Rlm6* was not introduced commercially after the French National Institute for Agricultural Research (INRA) decided to keep it as a research tool [41]. As a result, *Avrlm6* frequency was 100% in France [49], similar to Canada, where *Avrlm6* frequency exceeds 98% [45]. In our study, a fluctuation in frequency between 88% and 100% depending on the region was observed, however the use of *Rlm6* in Germany is not clear.

*Avrlm11* has been reported to occur in high frequencies in many regions of the world. For instance, its frequency was >95% in France and 100% in Canada [33,45]. In our study, the rate of isolates harboring *Avrlm11* ranged from 66% to 95%. Despite the high frequency of *Avrlm11*, it was found to be located on a dispensable mini-chromosome of *L. maculans* that was occasionally lost by meiosis. Thus, the loss of *Avrlm11* is related to the loss of this mini-chromosome in *L. maculans.* [33]. In spite of its dispensability, this chromosome turned out to be influential for the viability of ascospores, and thus, its loss has fitness costs [33].

The race structure in our study was described based on the phenotypic analysis of eight *Avr* genes and the PCR assays for *Avrlm6* and *Avrlm11*. Assuming each of the 10 tested *Avr* genes has a minimum of 2 alleles, the theoretical number of combinations would be 1024 (i.e., $2^{10}$). This shows the theoretical potential of the pathogen to break resistance. In our study, we were able to determine 52 races, the majority of which had a complexity of five *Avr* genes. Race complexity depicts the range of the available effective major *R* genes for setting management strategies for resistant cultivar rotation in a region [45]. In general, we found, according to our isolate collection, that the major *R* genes that provide sufficient resistance in German oilseed rape fields are *LepR1, LepR2, Rlm6, Rlm11* and *Rlm7,* while 61.3% of the isolates can equally infect cultivars harboring *Rlm1, Rlm2* and *Rlm4.* This is consistent with our previous investigation in Germany [46]. Combining quantitative resistance with race-specific resistance can be important to expand the durability of major *R* genes. It was demonstrated that *Rlm6* in a cultivar with a quantitative resistance background preserved its efficacy two years longer than in a cultivar with a susceptible background [41].

Pathogens with a high evolutionary potential are expected to overcome genetic resistance more rapidly [5]. It is believed that there are two important factors that enhance the evolutionary potential of *L. maculans*: first, the mixed reproduction systems, and second, the dispersal mechanisms. The sexual reproduction is a milestone for producing variations in the population, while the wind dispersal of spores spread the evolving races effectively over several kilometers [54,63]. However, in seasons when the environmental conditions do not favor sexual reproduction, asexual reproduction would be dominant, and thus, the ratio of idiomorphs might change. For example, in some Canadian regions, samples collected in 2010 and 2015 showed that the mating type *MAT1-2* was significantly more dominant than *MAT1-1* [64]. In our study, we showed that the idiomorphs of *L. maculans* in Germany did not significantly deviate from the ratio 1:1. This is consistent with the ratio observed in France in isolates collected in 2000–2003 [65] and in Canada in samples from 2011–2014 [64], which indicates similar lifecycles of this pathogen in different regions in the world and a central role of the sexual outcrossing in forming the population of the pathogen.

Overall, we comprehensively compared the effectiveness of major *R* genes in different countries. We found similarities in the *R* gene efficacies throughout Germany's neighboring countries, but increasingly significant differences the further those countries were from our study region. This may be related to the fact that the pathogen mainly spreads through wind-borne ascospores that can fly up to several kilometers and stay viable for six weeks [1]. We therefore recommend the consideration of geographical factors for the successful management of blackleg disease.

## 4. Materials and Methods

### 4.1. Sampling and Isolation of Leptosphaeria maculans

To explore the race spectrum of *Leptosphaeria maculans* in Germany, samples were collected from plots sown with the winter oilseed rape cultivar NK-Bravour (Syngenta Seeds GmbH, Bad Salzuflen, Germany). Plants grown in these plots were used as *L. maculans* trap plants, since this cultivar only harbors the major *R* gene *Rlm9* (H. Uphoff and M. Gundemann, Syngenta Seeds, pers. comm) [66]. The field trials were established in cooperation with breeding companies in seven regions of four provinces in Germany. Four fields were located in Lower Saxony in Einbeck (KWS), Nienstädt (Bayer CropScience), Peine (Limagrain) and Asendorf (DSV), one field in Saxony Anhalt in Hadmersleben (Syngenta), one field in Mecklenburg Western Pomerania in Groß Helle (NPZ) and one field in Schleswig Holstein in Sörup (BASF). Samples were collected in seasons 2017/2018, 2018/2019 and 2019/2020 (Table 4).

**Table 4.** Sampling seasons, regions and the numbers of *L. maculans* isolates.

| Season | Region | Province | No. of Isolates |
|---|---|---|---|
| 2017–2018 | Einbeck | Lower Saxony | 71 |
| 2017–2018 | Nienstädt | Lower Saxony | 99 |
| 2017–2018 | Hadmersleben | Saxony Anhalt | 90 |
| 2017–2018 | Groß Helle | Mecklenburg-Western Pomerania | 83 |
| 2018–2019 | Sörup | Schleswig-Holstein | 60 |
| 2019–2020 | Peine | Lower Saxony | 97 |
| 2019–2020 | Asendorf | Lower Saxony | 100 |
| | | Sum | 600 |

Leaf samples with characteristic phoma lesions were collected in autumn at growth stage BBCH 18 and in spring at growth stage BBCH 30 [67]. Samples were dried and stored at 4 °C until they were used for isolation. Isolation was conducted by incubating a dried leaf segment with a lesion in a humid chamber at room temperature to induce conidiation. Single pycnidium isolates were prepared by plating spores on synthetic Nutrient-Poor Agar (SNA) medium amended with 200 ppm streptomycin under sterile conditions. Petri dishes were incubated under NUV light at 20 °C for six days. Afterwards, a mycelial plug was transferred to a V8-juice medium supplemented with 200 ppm streptomycin and incubated for 10–14 days under the same conditions. Subsequently, spore suspensions were prepared and adjusted to a density of $1 \times 10^7$ spores/mL using a hemocytometer. Spore suspensions were stored at −20 °C and thawed just before they were used for inoculation.

To characterize *L. maculans* isolates, cotyledon tests were applied using a differential set of *B. napus* genotypes harboring the major *R* genes *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7*, *Rlm9*, *LepR1*, *LepR2* and *LepR3*. The cultivars Westar and Topas DH16516 had no major *R* genes and were used as susceptible controls (Table 5).

Seeds were sown in trays filled with potting soil and covered with transparent plastic plates to keep a high level of humidity. After three days, when seeds germinated, the covers were taken off. On the sixth day, seedlings were transplanted in multi-pot propagation trays with a 3:3:1 mixture of garden soil, compost and sand. On day seven, seedlings were inoculated with 10 µL of spore suspension placed on each lobe of the cotyledons after injuring it with a needle. Seedlings were then put under controlled conditions of 16:8 h light (day/night) at 20 °C. For each isolate and differential line, eight seedlings were inoculated. Symptoms were evaluated 14 days post-inoculation according to the IMASCORE rating scale, where class one shows typical hypersensitive reactions and class six reflects tissue collapse with sporulation. Classes one to three are considered as incompatible reactions while classes four to six are noted as compatible ones [72].

**Table 5.** Differential sets of *B. napus* cultivars or introgression lines used for race typing of *L. maculans* isolates.

| Cultivar/Line | *R* gene | References |
|---|---|---|
| Westar [a] | No *R* gene | Balesdent et al., 2002 [68] |
| Columbus [a] | *Rlm1*, *Rlm3* | Balesdent et al., 2006 [49] |
| Bristol [a] | *Rlm2*, *Rlm9* | Balesdent et al., 2006 [49] |
| 02–22-2-1 [a] | *Rlm3* | Delourme, 2012 [69] |
| Jet Neuf [a] | *Rlm4* | Balesdent et al., 2006 [49] |
| 01-23-2-1 [a] | *Rlm7* | Delourme, 2012 [69] |
| Caiman [a] | *Rlm7* | Marcoft et al., 2012 [70] |
| Goéland [a] | *Rlm9* | Balesdent et al., 2006 [49] |
| Topas DH16516 [b] | No *R* gene | Larkan et al., 2016 [71] |
| Topas-*Rlm1* [b] | *Rlm1* | Larkan et al., 2016 [71] |
| Topas-*Rlm2* [b] | *Rlm2* | Larkan et al., 2016 [71] |
| Topas-*Rlm3* [b] | *Rlm3* | Larkan et al., 2016 [71] |
| Topas-*Rlm4* [b] | *Rlm4* | Larkan et al., 2016 [71] |
| Topas-*LepR1* [b] | *LepR1* | Larkan et al., 2016 [71] |
| Topas-*LepR2* [b] | *LepR2* | Larkan et al., 2016 [71] |
| Topas-*LepR3* [b] | *LepR3* | Larkan et al., 2016 [71] |

[a] Provided by Dr. R. Delourme, Institute for Genetics, Environment and Plant Protection, INRA, Rennes, France. [b] Provided by Dr. Hossein Borhan and Dr. Nicholas Larkan, Agriculture and Agri-Food Canada, Saskatoon, Canada.

### *4.2. DNA Extraction and PCR Assays*

DNA templates were prepared using a simple boiling DNA extraction method. Shortly, 100 µL of spore suspension ($10^7$ spores/mL) was centrifuged at 16,000× *g* for 10 min. Afterwards, the supernatant was discarded carefully to keep the pellet. Fifty microliters of Tris (10 mM) was added to the pellet and homogenized by vortexing. The suspension was exposed to an ultrasound for 5 s and then transferred to a water bath at 98 °C for 10 min. Subsequently, the tubes were put on ice for 10 min. Before applying the PCR tests, the quality and quantity of the extracts were checked using agarose gel electrophoresis (0.8% (*w*/*v*)) supplied with Midori Green (NIPPON Genetics Europe GmbH). Five µL of DNA was mixed with two µL of the loading dye (100 mM EDTA, 50% (*v*/*v*) glycerol and 0.025% (*w*/*v*) bromophenol-blue) and run in TBE buffer at 3 V/cm for 60 min. DNA quantity was afterwards compared with a standard Lambda phage DNA of concentrations ranging from 150 to 35.5 ng. The concept of releasing DNA by boiling the cells was also used by Adwan [73].

To distinguish *L. maculans* and *L. biglobosa*, we amplified the internal transcribed spacer (ITS) regions of the pathogen using ITS4 and ITS5 primers (Table 6). The steps of the 35 PCR thermal cycles are described in Table 7. *Avrlm6* and *Avrlm11* were amplified using the primer pairs listed in Table 6 in 40 and 30 thermal cycles, respectively. PCR profiles are shown in Table 7. In general, the end volume of a PCR reaction was 25 µL, of which 7 µL was DNA extract. The mixture contained 1 µM of each primer pair, except for the mating type primers, where 0.67 µM of each of the three primers were used. The concentration of the nucleoside triphosphates (dNTPs) was 0.2 mM, and 1 unit of the FastGene Taq DNA polymerase (NIPPON Genetics Europe GmbH) was added. The reaction was conducted in a buffer containing 1.5 mM of $MgCl_2$. PCR reactions were conducted in a T Professional Basic Gradient thermal cycler (Biometra, Göttingen, Germany).

**Table 6.** Sequences of primers used in this study.

| Primer | Sequence (5 to 3) | References |
|---|---|---|
| *Avrlm6*-F | TCAATTTGTCTGTTCAAGTTATGGA | Fudal et al., 2009 [74] |
| *Avrlm6*-R | CCAGTTTTGAACCGTAGAGGTAGCA | Fudal et al., 2009 [74] |
| *Avrlm11*-F | TGCGTTTCTTGCTTCCTATATTT | Balesdent et al., 2013 [33] |
| *Avrlm11*-R | CAAGTTGGATCTTTCTCATTCG | Balesdent et al., 2013 [33] |
| *MAT* Locus | TGGCGAATTAAGGGATTGCTG | Cozijnsen and Howlett, 2003 [11] |
| *MAT1-1* | CTCGATGCAATGTACTTGG | Cozijnsen and Howlett, 2003 [11] |
| *MAT1-2* | AGCCGGAGGTGAAGTTGAAGCCG | Cozijnsen and Howlett, 2003 [11] |
| ITS4 | TCCTCCGCTTATTGATATGC | White et al., 1990 [75] |
| ITS5 | GGAAGTAAAAGTCGTAACAAGG | White et al., 1990 [75] |

**Table 7.** Profiles of the individual PCR assays.

| Target | Initial Denaturation | Denaturation | Annealing | Extension | No. of Cycles |
|---|---|---|---|---|---|
| ITS | 95 °C | 94 °C | 57 °C | 72 °C | 35 |
| *Avrlm6* | 95 °C | 94 °C | 60 °C | 72 °C | 40 |
| *Avrlm11* | 95 °C | 94 °C | 59 °C | 72 °C | 30 |
| *MAT1-1*/*MAT1-2* | 95 °C | 94 °C | 60 °C | 72 °C | 35 |

To visualize the PCR products, 5 µL was mixed with 2 µL of the loading dye described above. The mixture was loaded on 1% agarose gel supplied with Midori Green. Electrophoresis was run at 3 V/cm for 60 min for all PCR products, except for the mating type tests, where the electrophoresis was run for at least two hours. Mating types of *L. maculans* were defined using a multiplex PCR, as described by Cozijnsen and Howlett [11]. An isolate of *L. maculans* has a single mating locus (*MAT* locus) and one of the two loci *MAT1-1* or *MAT1-2*. The common primer for the *MAT* locus in combination with the *MAT1-1*-specific primer resulted in an amplicon of 686 bp, while the common primer in combination with the *MAT1-2*-specific primer afforded an amplicon of 443 bp.

### *4.3. Statistical Analysis*

For phenotypic characterization of the *L. maculans* population, the numbers of compatible (susceptible) reactions observed in the cotyledon tests were represented relative to the number of isolates collected per region. Similarly, *Avrlm6* and *Avrlm11* that produced bands in PCR tests were represented as the proportion of the total number of isolates analyzed per region. The Margalef index ($D_{Mg}$) was calculated using the following formula:

$$D_{Mg} = (S - 1)/\mathrm{Ln}(N)$$

where S is the number of races per region, and N is the number of *L. maculans* isolates in the corresponding region.

Fisher's exact test $\chi^2$ was applied using the software R [76] to test the null hypothesis that the mating types of *L. maculans* in German fields do not deviate from the ratio 1:1 at a confidence level of 95%.

## 5. Conclusions

In this study, the *L. maculans* population in German oilseed rape fields was characterized by their races. The resulting *Avr* profile of the pathogen provides an important basis for evaluating the state of efficacy of major *R* genes used in commercial cultivars. Such knowledge is a prerequisite for establishing a management strategy with the aim of protecting *R* genes from a premature decline in efficacy and for expanding their durability in practical use. The rapid breakdown of major *R* genes identified in this study strongly implies the need for combining resistant cultivars with other control measures. Considering the long breeding cycles of 7–10 years required to have a new major *R* gene established in

a registered new cultivar, it is important to combine quantitative resistance with major *R* genes in order to achieve more durable and sustainable disease control. The similarities observed in *Avr* profiles of *L. maculans* between France and Germany suggest that such management strategies can be valuable on a broader geographical scale. In addition, different *Avr* profiles existing in European and Canadian oilseed rape fields imply that transfer of races between continents by seed trade must be avoided. Hence, we emphasized the importance of considering *L. maculans* in the phytosanitary measures, ensuring international clean seed pathways.

**Author Contributions:** Conceptualization, B.K. and D.A.; methodology, D.A.; validation D.A.; formal analysis, D.A.; investigation, D.A.; data curation, D.A.; writing—original draft preparation, D.A.; writing, reviewing and editing, B.K. and A.v.T.; visualization, D.A.; supervision, B.K. and A.v.T.; project administration, B.K.; funding acquisition, B.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Federal Ministry of Food and Agriculture, Germany, grant number 2818205015.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** We thank Regine Delourme (INRA Rennes), Hossein Borhan and Nicholas Larkan (AAFC Canada) for providing the differential lines of *B. napus*. We also appreciate the excellent technical assistance by Jutta Schaper, Heike Rollwage, Evelin Vorbeck and Isabel Müller. We also acknowledge the efforts contributed by the bachelor students Justin Fricke and Elisabeth Schrade and the trainees Luan Vitor Nascimento and Anam Rashid in the greenhouse experiments and PCR tests.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## References

1. West, J.S.; Kharbanda, P.D.; Barbetti, M.J.; Fitt, B.D.L. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathol.* **2001**, *50*, 10–27. [CrossRef]
2. Shoemaker, R.A.; Brun, H. The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. *Can. J. Bot.* **2001**, *79*, 412–419. [CrossRef]
3. Mendes-Pereira, E.; Balesdent, M.-H.; Brun, H.; Rouxel, T. Molecular phylogeny of the *Leptosphaeria maculans—L. biglobosa* species complex. *Mycol. Res.* **2003**, *107*, 1287–1304. [CrossRef] [PubMed]
4. Stonard, J.F.; Latunde-Dada, A.O.; Huang, Y.-J.; West, J.S.; Evans, N.; Fitt, B.D.L. Geographic variation in severity of phoma stem canker and *Leptosphaeria maculans/L. biglobosa* populations on UK winter oilseed rape (*Brassica napus*). *Eur. J. Plant Pathol.* **2010**, *126*, 97–109. [CrossRef]
5. McDonald, B.A.; Linde, C. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* **2002**, *40*, 349–379. [CrossRef]
6. Kaczmarek, J.; Jędryczka, M. Characterization of two coexisting pathogen populations of *Leptosphaeria* spp., the cause of stem canker of brassicas. *Acta Agrobot.* **2012**, *64*, 3–14. [CrossRef]
7. Fernando, W.G.D.; Zhang, X.; Amarasinghe, C.C. Detection of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* causing blackleg disease in canola from Canadian canola seed lots and dockage. *Plants* **2016**, *5*, 12. [CrossRef]
8. Mahuku, G.S.; Goodwin, P.H.; Hall, R.; Hsiang, T. Variability in the highly virulent type of *Leptosphaeria maculans* within and between oilseed rape fields. *Can. J. Bot.* **1997**, *75*, 1485–1492. [CrossRef]
9. Barrins, J.M.; Ades, P.K.; Salisbury, P.A.; Howlett, B.J. Genetic diversity of Australian isolates of *Leptosphaeria maculans*, the fungus that causes blackleg of canola (*Brassica napus*). *Australas. Plant Pathol.* **2004**, *33*, 529. [CrossRef]
10. Ghanbarnia, K.; Dilantha Fernando, W.G.; Crow, G. Comparison of disease severity and incidence at different growth stages of naturally infected canola plants under field conditions by pycnidiospores of *Phoma lingam* as a main source of inoculum. *Can. J. Plant Pathol.* **2011**, *33*, 355–363. [CrossRef]
11. Cozijnsen, A.J.; Howlett, B.J. Characterisation of the mating-type locus of the plant pathogenic ascomycete *Leptosphaeria maculans*. *Curr. Genet.* **2003**, *43*, 351–357. [CrossRef]

12. Milgroom, M.G. Recombination and the multilocus structure of fungal populations. *Annu. Rev. Phytopathol.* **1996**, *34*, 457–477. [CrossRef]
13. Deb, D.; Khan, A.; Dey, N. Phoma diseases: Epidemiology and control. *Plant Pathol.* **2020**, *69*, 1203–1217. [CrossRef]
14. Kutcher, H.R.; Brandt, S.A.; Smith, E.G.; Ulrich, D.; Malhi, S.S.; Johnston, A.M. Blackleg disease of canola mitigated by resistant cultivars and four-year crop rotations in western Canada. *Can. J. Plant Pathol.* **2013**, *35*, 209–221. [CrossRef]
15. Pilet, M.L.; Delourme, R.; Foisset, N.; Renard, M. Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculans* (Desm.) Ces. et de Not., in Winter rapeseed (*Brassica napus* L.). *Theor. Appl. Genet.* **1998**, *96*, 23–30. [CrossRef]
16. Jones, J.D.G.; Dangl, J.L. The plant immune system. *Nature* **2006**, *444*, 323–329. [CrossRef]
17. Flor, H.H. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* **1971**, *9*, 275–296. [CrossRef]
18. Agrios, G.N. *Plant Pathology*, 5th ed.; Elsevier Science: Burlington, VT, USA, 2005; ISBN 9780120445653.
19. Keen, N.T. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* **1990**, *24*, 447–463. [CrossRef]
20. Thomma, B.P.H.J.; Nürnberger, T.; Joosten, M.H.A.J. Of PAMPs and effectors: The blurred PTI-ETI dichotomy. *Plant Cell* **2011**, *23*, 4–15. [CrossRef]
21. Chisholm, S.T.; Coaker, G.; Day, B.; Staskawicz, B.J. Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* **2006**, *124*, 803–814. [CrossRef]
22. Balesdent, M.H.; Barbetti, M.J.; Li, H.; Sivasithamparam, K.; Gout, L.; Rouxel, T. Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathology* **2005**, *95*, 1061–1071. [CrossRef]
23. Van de Wouw, A.P.; Marcroft, S.J.; Barbetti, M.J.; Hua, L.; Salisbury, P.A.; Gout, L.; Rouxel, T.; Howlett, B.J.; Balesdent, M.H. Dual control of avirulence in *Leptosphaeria maculans* towards a *Brassica napus* cultivar with 'sylvestris -derived' resistance suggests involvement of two resistance genes. *Plant Pathol.* **2009**, *58*, 305–313. [CrossRef]
24. Larkan, N.J.; Lydiate, D.J.; Parkin, I.A.P.; Nelson, M.N.; Epp, D.J.; Cowling, W.A.; Rimmer, S.R.; Borhan, M.H. The *Brassica napus* blackleg resistance gene *LepR3* encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector *Avrlm1*. *New Phytol.* **2013**, *197*, 595–605. [CrossRef]
25. Petit-Houdenot, Y.; Degrave, A.; Meyer, M.; Blaise, F.; Ollivier, B.; Marais, C.-L.; Jauneau, A.; Audran, C.; Rivas, S.; Veneault-Fourrey, C.; et al. A two genes–for–one gene interaction between *Leptosphaeria maculans* and *Brassica napus*. *New Phytol.* **2019**, *223*, 397–411. [CrossRef]
26. Plissonneau, C.; Daverdin, G.; Ollivier, B.; Blaise, F.; Degrave, A.; Fudal, I.; Rouxel, T.; Balesdent, M.-H. A game of hide and seek between avirulence genes *AvrLm4-7* and *AvrLm3* in *Leptosphaeria maculans*. *New Phytol.* **2016**, *209*, 1613–1624. [CrossRef]
27. Ghanbarnia, K.; Ma, L.; Larkan, N.J.; Haddadi, P.; Fernando, W.G.D.; Borhan, M.H. *Leptosphaeria maculans AvrLm9*: A new player in the game of hide and seek with *AvrLm4-7*. *Mol. Plant Pathol.* **2018**, *19*, 1754–1764. [CrossRef]
28. Gout, L.; Fudal, I.; Kuhn, M.-L.; Blaise, F.; Eckert, M.; Cattolico, L.; Balesdent, M.-H.; Rouxel, T. Lost in the middle of nowhere: The *AvrLm1* avirulence gene of the Dothideomycete *Leptosphaeria maculans*. *Mol. Microbiol.* **2006**, *60*, 67–80. [CrossRef]
29. Ghanbarnia, K.; Fudal, I.; Larkan, N.J.; Links, M.G.; Balesdent, M.-H.; Profotova, B.; Fernando, W.G.D.; Rouxel, T.; Borhan, M.H. Rapid identification of the *Leptosphaeria maculans* avirulence gene *AvrLm2* using an intraspecific comparative genomics approach. *Mol. Plant Pathol.* **2015**, *16*, 699–709. [CrossRef]
30. Parlange, F.; Daverdin, G.; Fudal, I.; Kuhn, M.-L.; Balesdent, M.-H.; Blaise, F.; Grezes-Besset, B.; Rouxel, T. *Leptosphaeria maculans* avirulence gene *AvrLm4-7* confers a dual recognition specificity by the *Rlm4* and *Rlm7* resistance genes of oilseed rape, and circumvents *Rlm4*-mediated recognition through a single amino acid change. *Mol. Microbiol.* **2009**, *71*, 851–863. [CrossRef]
31. Van de Wouw, A.P.; Lowe, R.G.T.; Elliott, C.E.; Dubois, D.J.; Howlett, B.J. An avirulence gene, *AvrLmJ1*, from the blackleg fungus, *Leptosphaeria maculans*, confers avirulence to *Brassica juncea* cultivars. *Mol. Plant Pathol.* **2014**, *15*, 523–530. [CrossRef]
32. Fudal, I.; Ross, S.; Gout, L.; Blaise, F.; Kuhn, M.L.; Eckert, M.R.; Cattolico, L.; Bernard-Samain, S.; Balesdent, M.H.; Rouxel, T.; et al. Heterochromatin-like regions as ecological niches for avirulence genes in the *Leptosphaeria maculans* genome: Map-based cloning of *AvrLm6*. *Mol. Plant Microbe Interact.* **2007**, *20*, 459–470. [CrossRef] [PubMed]
33. Balesdent, M.-H.; Fudal, I.; Ollivier, B.; Bally, P.; Grandaubert, J.; Eber, F.; Chèvre, A.-M.; Leflon, M.; Rouxel, T. The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *Brassica rapa*. *New Phytol.* **2013**, *198*, 887–898. [CrossRef] [PubMed]
34. Yu, F.; Lydiate, D.J.; Rimmer, S.R. Identification of two novel genes for blackleg resistance in *Brassica napus*. *Theor. Appl. Genet.* **2005**, *110*, 969–979. [CrossRef] [PubMed]
35. Yu, F.; Lydiate, D.J.; Rimmer, S.R. Identification and mapping of a third blackleg resistance locus in *Brassica napus* derived from *B. rapa* subsp. sylvestris. *Genome* **2008**, *51*, 64–72. [CrossRef]
36. Yu, F.; Gugel, R.K.; Kutcher, H.R.; Peng, G.; Rimmer, S.R. Identification and mapping of a novel blackleg resistance locus *LepR4* in the progenies from *Brassica napus* × *B. rapa* subsp. sylvestris. *Theor. Appl. Genet.* **2013**, *126*, 307–315. [CrossRef]
37. Delourme, R.; Chèvre, A.M.; Brun, H.; Rouxel, T.; Balesdent, M.H.; Dias, J.S.; Salisbury, P.; Renard, M.; Rimmer, S.R. Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). In *Sustainable Strategies for Managing Brassica napus (Oilseed Rape) Resistance to Leptosphaeria maculans (Phoma Stem Canker)*; Fitt, B.D.L., Evans, N., Howlett, B.J., Cooke, B.M., Eds.; Springer: Berlin/Heidelberg, Germany, 2006; pp. 41–52. ISBN 1-4020-4524-7.

38. Long, Y.; Wang, Z.; Sun, Z.; Fernando, D.W.G.; McVetty, P.B.E.; Li, G. Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola cultivar 'Surpass 400'. *Theor. Appl. Genet.* **2011**, *122*, 1223–1231. [CrossRef]
39. Larkan, N.J.; Ma, L.; Borhan, M.H. The *Brassica napus* receptor-like protein *Rlm2* is encoded by a second allele of the *LepR3/Rlm2* blackleg resistance locus. *Plant Biotechnol. J.* **2015**, *13*, 983–992. [CrossRef]
40. Raman, H.; Raman, R.; Qiu, Y.; Zhang, Y.; Batley, J.; Liu, S. The *Rlm13* Gene, a New Player of *Brassica napus-Leptosphaeria maculans* Interaction Maps on Chromosome C03 in Canola. *Front. Plant Sci.* **2021**, *12*, 675. [CrossRef]
41. Brun, H.; Chèvre, A.-M.; Fitt, B.D.L.; Powers, S.; Besnard, A.-L.; Ermel, M.; Huteau, V.; Marquer, B.; Eber, F.; Renard, M.; et al. Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytol.* **2010**, *185*, 285–299. [CrossRef]
42. Rouxel, T.; Penaud, A.; Pinochet, X.; Brun, H.; Gout, L.; Delourme, R.; Schmit, J.; Balesdent, M.-H. A 10-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the *Rlm1* resistance gene of oilseed rape. *Eur. J. Plant Pathol.* **2003**, *109*, 871–881. [CrossRef]
43. Sprague, S.J.; Balesdent, M.-H.; Brun, H.; Hayden, H.L.; Marcroft, S.J.; Pinochet, X.; Rouxel, T.; Howlett, B.J. Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. *Eur. J. Plant Pathol.* **2006**, *114*, 33–40. [CrossRef]
44. Zhang, X.; Peng, G.; Kutcher, H.R.; Balesdent, M.-H.; Delourme, R.; Fernando, W.G.D. Breakdown of *Rlm3* resistance in the *Brassica napus–Leptosphaeria maculans* pathosystem in western Canada. *Eur. J. Plant Pathol.* **2016**, *145*, 659–674. [CrossRef]
45. Cornelsen, J.; Zou, Z.; Huang, S.; Parks, P.; Lange, R.; Peng, G.; Fernando, W.G.D. Validating the strategic deployment of blackleg resistance gene groups in commercial canola fields on the Canadian prairies. *Front. Plant Sci.* **2021**, *12*, 669997. [CrossRef]
46. Winter, M.; Koopmann, B. Race spectra of *Leptosphaeria maculans*, the causal agent of blackleg disease of oilseed rape, in different geographic regions in northern Germany. *Eur. J. Plant Pathol.* **2016**, *145*, 629–641. [CrossRef]
47. Van de Wouw, A.P.; Howlett, B.J. Advances in understanding the *Leptosphaeria maculans-Brassica* pathosystem and their impact on disease management. *Can. J. Plant Pathol.* **2020**, *42*, 149–163. [CrossRef]
48. Dolatabadian, A.; Cornelsen, J.; Huang, S.; Zou, Z.; Fernando, W.D. Sustainability on the farm: Breeding for resistance and management of major canola diseases in Canada contributing towards an IPM approach. *Can. J. Plant Pathol.* **2021**, *44*, 157–190. [CrossRef]
49. Balesdent, M.-H.; Louvard, K.; Pinochet, X.; Rouxel, T. A Large-scale survey of races of *Leptosphaeria maculans* occurring on oilseed rape in France. *Eur. J. Plant Pathol.* **2006**, *114*, 53–65. [CrossRef]
50. Soomro, W.; Kutcher, R.; Yu, F.; Hwang, S.-F.; Fernando, D.; Strelkov, S.E.; Peng, G. The race structure of *Leptosphaeria maculans* in western Canada between 2012 and 2014 and its influence on blackleg of canola. *Can. J. Plant Pathol.* **2021**, *43*, 480–493. [CrossRef]
51. Plissonneau, C.; Blaise, F.; Ollivier, B.; Leflon, M.; Carpezat, J.; Rouxel, T.; Balesdent, M.-H. Unusual evolutionary mechanisms to escape effector-triggered immunity in the fungal phytopathogen *Leptosphaeria maculans*. *Mol. Ecol.* **2017**, *26*, 2183–2198. [CrossRef]
52. Lazar, N.; Mesarich, C.H.; Petit-Houdenot, Y.; Talbi, N.; de La Sierra-Gallay, I.L.; Zélie, E.; Blondeau, K.; Gracy, J.; Ollivier, B.; Blaise, F.; et al. A new family of structurally conserved fungal effectors displays epistatic interactions with plant resistance proteins. *bioRxiv* **2020**. [CrossRef]
53. Guttman, D.S.; McHardy, A.C.; Schulze-Lefert, P. Microbial genome-enabled insights into plant-microorganism interactions. *Nat. Rev. Genet.* **2014**, *15*, 797–813. [CrossRef]
54. Daverdin, G.; Rouxel, T.; Gout, L.; Aubertot, J.-N.; Fudal, I.; Meyer, M.; Parlange, F.; Carpezat, J.; Balesdent, M.-H. Genome structure and reproductive behaviour influence the evolutionary potential of a fungal phytopathogen. *PLoS Pathog.* **2012**, *8*, e1003020. [CrossRef]
55. Gout, L.; Kuhn, M.L.; Vincenot, L.; Bernard-Samain, S.; Cattolico, L.; Barbetti, M.; Moreno-Rico, O.; Balesdent, M.-H.; Rouxel, T. Genome structure impacts molecular evolution at the *AvrLm1* avirulence locus of the plant pathogen *Leptosphaeria maculans*. *Environ. Microbiol.* **2007**, *9*, 2978–2992. [CrossRef]
56. Van de Wouw, A.P.; Stonard, J.F.; Howlett, B.J.; West, J.S.; Fitt, B.D.L.; Atkins, S.D. Determining frequencies of avirulent alleles in airborne *Leptosphaeria maculans* inoculum using quantitative PCR. *Plant Pathol.* **2010**, *59*, 809–818. [CrossRef]
57. Stachowiak, A.; Olechnowicz, J.; Jedryczka, M.; Rouxel, T.; Balesdent, M.-H.; Happstadius, I.; Gladders, P.; Latunde-Dada, A.; Evans, N. Frequency of avirulence alleles in field populations of *Leptosphaeria maculans* in Europe. *Eur. J. Plant Pathol.* **2006**, *114*, 67–75. [CrossRef]
58. Balesdent, M.-H.; Plissonneau, C.; Coudard, L.; Daverdin, G.; Le Meur, L.; Carpezat, J.; Leflon, M.; Pinochet, X.; Ermel, M.; Brun, H.; et al. Résistance du colza au phoma: Où en est l'efficacité de Rlm7? *Phytoma la Défense des Végétaux* **2015**, *Mai (684)*, 20–24.
59. Chèvre, A.M.; Barret, P.; Eber, F.; Dupuy, P.; Brun, H.; Tanguy, X.; Renard, M. Selection of stable *Brassica napus-B. juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*). 1. Identification of molecular markers, chromosomal and genomic origin of the introgression. *Theor. Appl. Genet.* **1997**, *95*, 1104–1111. [CrossRef]
60. Bousset, L.; Ermel, M.; Delourme, R. A *Leptosphaeria maculans* set of isolates characterised on all available differentials and used as control to identify virulence frequencies in a current French population. *bioRxiv* **2020**. [CrossRef]
61. KWS SAAT SE & Co. KGaA. ADELMO KWS. Available online: https://www.kws.com/fr/fr/produits/colza/adelmo-kws/ (accessed on 28 December 2021).

62. Van de Wouw, A.P.; Howlett, B.J.; Idnurm, A. Changes in allele frequencies of avirulence genes in the blackleg fungus, *Leptosphaeria maculans*, over two decades in Australia. *Crop Pasture Sci.* **2018**, *69*, 20. [CrossRef]
63. McGee, D.C. Black leg (*Leptosphaeria maculans* (Desm.) Ces. de Not.) of rapeseed in Victoria: Sources of infection and relationships between inoculum, environmental factors and disease severity. *Aust. J. Agric. Res.* **1977**, *28*, 53. [CrossRef]
64. Fernando, W.G.D.; Zhang, X.; Selin, C.; Zou, Z.; Liban, S.H.; McLaren, D.L.; Kubinec, A.; Parks, P.S.; Rashid, M.H.; Padmathilake, K.R.E.; et al. A six-year investigation of the dynamics of avirulence allele profiles, blackleg incidence, and mating type alleles of *Leptosphaeria maculans* populations associated with canola crops in Manitoba, Canada. *Plant Dis.* **2018**, *102*, 790–798. [CrossRef] [PubMed]
65. Gout, L.; Eckert, M.; Rouxel, T.; Balesdent, M.-H. Genetic variability and distribution of mating type alleles in field populations of *Leptosphaeria maculans* from France. *Appl. Environ. Microbiol.* **2006**, *72*, 185–191. [CrossRef] [PubMed]
66. Uphoff, H.; Gundmann, M.; (Syngenta Seeds GmbH, Bad Salzuflen, Germany). Personal communication, 2022.
67. Lancashire, P.D.; Bleiholder, H.; Boom, T.V.D.; Langelüddeke, P.; Stauss, R.; Weber, E.; Witzenberger, A. A Uniform decimal code for growth stages of crops and weeds. *Ann. Appl. Biol.* **1991**, *119*, 561–601. [CrossRef]
68. Balesdent, M.H.; Attard, A.; Kühn, M.L.; Rouxel, T. New Avirulence Genes in the Phytopathogenic Fungus *Leptosphaeria maculans*. *Phytopathology* **2002**, *92*, 1122–1133. [CrossRef] [PubMed]
69. Delourme, R.; (INRA, Le Rheu, France). Personal communication, 2012.
70. Marcroft, S.J.; Elliott, V.L.; Cozijnsen, A.J.; Salisbury, P.A.; Howlett, B.J.; van de Wouw, A.P. Identifying resistance genes to *Leptosphaeria maculans* in Australian *Brassica napus* cultivars based on reactions to isolates with known avirulence genotypes. *Crop Pasture Sci.* **2012**, *63*, 338–350. [CrossRef]
71. Larkan, N.J.; Yu, F.; Lydiate, D.J.; Rimmer, S.R.; Borhan, M.H. Single R Gene introgression lines for accurate dissection of the *Bra–sica-Leptosphaeria* Pathosystem. *Front. Plant Sci.* **2016**, *7*, 1771. [CrossRef]
72. Balesdent, M.H.; Attard, A.; Ansan-Melayah, D.; Delourme, R.; Renard, M.; Rouxel, T. Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans*. *Phytopathology* **2001**, *91*, 70–76. [CrossRef]
73. Adwan, K. Fast DNA isolation and PCR protocols for detection of methicillin-resistant staphylococci. *Folia Microbiol.* **2014**, *59*, 5–8. [CrossRef]
74. Fudal, I.; Ross, S.; Brun, H.; Besnard, A.-L.; Ermel, M.; Kuhn, M.-L.; Balesdent, M.-H.; Rouxel, T. Repeat-induced point mutation (RIP) as an alternative mechanism of evolution toward virulence in *Leptosphaeria maculans*. *Mol. Plant Microbe Interact.* **2009**, *22*, 932–941. [CrossRef]
75. White, T.J.; Bruns, T.; Lee, S.; Taylor, J.W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press, Inc.: New York, NY, USA, 1990; pp. 315–322. ISBN 0-12-372180-6.
76. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2020; Available online: https://www.r-project.org/ (accessed on 28 September 2021).

# Phenotyping of quantitative resistance to blackleg disease in a *B. napus* interconnected multiparent mapping population under greenhouse and field conditions

Dima Alnajar and Birger Koopmann

## Abstract

*Leptosphaeria maculans*, the causal agent of blackleg disease, is one of the main pathogens of oilseed rape. Two types of host resistance are generally described, qualitative resistance and quantitative resistance. Despite its partial effect, quantitative resistance is known to be more durable and stable compared to qualitative resistance. In this study, we compared four different inoculation methods to assess quantitative resistance under controlled conditions. The plants were inoculated either by spraying with spore suspension $10^7$ spores/ml on the (1) leaf upper side, or (2) leaf lower side, or by mycelial agar plug application, (3) directly on the petiole, or (4) at the stem base. Results showed that inoculation with a mycelial agar plug on an injured stem base is the most repeatable and reliable method. Using this inoculation method, we further evaluated 350 DH lines of interconnected multiparent mapping (IMM) oilseed rape population for quantitative resistance against blackleg disease under greenhouse conditions. Plants were assessed at 14, 35 and 49 dpi. On 49 dpi, both the area under the disease progress curve (AUDPC) and the volume of diseased tissues (VDT) were calculated. Results showed distinct variation among the tested DH lines. These results were further used to define QTLs that contributed to quantitative resistance (Chapter IV). Additionally, this study aimed to phenotype the IMM population for quantitative resistance to blackleg disease in the field at the growth stage BBCH80-83. However, our field study experienced abiotic and biotic challenges. Drought and heat waves resulted in the complete failure of one season. Wild boar also raided the crop in one field and caused significant damage. Besides, the insects' damage of plant stems complicated the monitoring process of blackleg. The larvae of the rape stem weevil fed on oilseed rape stems forming deep tunnels which in turn resulted in biased results when lesion depth caused by *L. maculans* was assessed in the stem. All sampled plants showed damage despite the application of the neonicotinoid systemic insecticide Biscaya. Overall, this study provided: a reliable method for phenotyping under greenhouse conditions; a database used to unravel new QTLs responsible for quantitative resistance in oilseed rape; and shed light on serious constraints for phenotyping quantitative resistance to *L. maculans* under natural conditions.

# 1. Introduction

*Brassica napus* is the second most economically important oilseed crop worldwide (Zheng et al. 2020). Several biotic and abiotic limiting factors affect its global production (Elferjani and Soolanayakanahally, 2018; Zheng et al. 2020). It has been recently reported that oilseed rape production witnessed a significant decline in the most important production areas due to extreme heat waves, especially in Canada (Nickel, 2021). Biotic stresses such as rape stem weevil, Sclerotinia stem rot and blackleg disease are among the most devastating production constraints. Blackleg disease (Phoma stem canker) is one of the top five biotic stresses of oilseed rape in Europe, Australia and Canada. While chemical, biological and cultural control methods of blackleg disease are available (Zheng et al. 2020), the use of resistant cultivars is considered one of the most effective and sustainable approaches (Fortune et al. 2021).

Two types of resistance against plant diseases are distinguished, qualitative resistance and quantitative disease resistance (QDR) (Poland et al. 2009). Due to its non-race specific character, QDR against *L. maculans* is believed to be more durable and more effective against newly evolving pathotypes compared to qualitative resistance (Parlevliet, 2002; McDonald and Linde, 2002). It was also demonstrated that pyramiding QTLs with major *R* genes expands their durability so that disease severity remains mild even if a major *R* gene efficacy decreases. Thus, production does not experience a sudden collapse with the emergence of novel pathogen races (Brun et al. 2010; Delourme et al. 2014). There are not enough discoveries of new alternative major *R* genes to compensate for the rapid loss of the *R* gene efficacies. This makes seeking new sources of QDR against blackleg disease more indispensable than ever. Therefore, researchers have recently increasingly invested in studies related to QDR against *L. maculans* in *B. napus* (e.g Jestin et al. 2015; Y. J. Huang et al. 2016; Rahman et al. 2016; Vollrath et al. 2021).

To identify genetic loci responsible for QDR, several analytical methods are used such as QTL mapping and genome-wide association studies (Gupta et al. 2019). However, all these approaches depend on phenotyping data produced by evaluating diversity in a quantitative trait in a genetically variable population (Cobb et al. 2013; Amas et al. 2021). Some studies on QDR against blackleg disease in oilseed rape used a collection of genetic accessions of oilseed rape (Jestin et al. 2011; Fikere et al. 2020). Others depended on either the F2 and F3 ($F_{2,3}$) or the DH lines produced from crossing different parental lines (Jestin et al. 2015; Huang et al. 2016). DH lines have the advantage of being genetically uniform. Thus, assessment results show more reproducibility when evaluated in different environments (Pink et al. 2008; Amas et al. 2021). QTL genetic mapping populations used to be produced in a traditional way by crossing two parental cultivars. There is however a risk that the detected QTLs are relevant

only to the cross used in the population and that the markers derived from it are not suitable to use in commercial cultivars (Obermeier and Friedt, 2015). Therefore, interconnected multiparent populations believed to give more reliable QTLs. Justin et al. (2015), identified new QTLs responsible for QDR against blackleg disease in a *B. napus* interconnected multiparent mapping population that resulted from crossing four resistant lines with a susceptible parent.

Mapping populations can be screened under controlled conditions or in the field (Jestin et al. 2015; Huang et al. 2019). Yet, even if QTLs were defined based on the assessment of a mapping population under controlled conditions, it is important to further ensure their consistency under various natural conditions, particularly as different agronomical practices, climatic conditions and pathogen race structures enhance the sensitivity of QDR to genotype × environment interactions (Amas et al. 2021). Increasing phenotyping throughput is a main challenge for the identification of QTLs associated with QDR against *L. maculans* (Amas et al. 2021). In general, phenotyping has two pillars: the inoculation methods and the art of the symptoms screened and the related scoring approaches. Under controlled conditions, ascospores or pycnidiospores have been used as inoculum (Huang et al. 2014). Seedlings can be exposed to an ascospore shower from infected stubble to mimic the natural conditions to assess QDR in planta (Marcroft et al. 2012). With other inoculation methods inoculum can be applied directly on young plants at the 2-3 leaf stage either on the petioles or on the leaf lamina (Huang et al. 2014). Assessing the disease can be visual or molecular. The visual assessment might depend on plant survival rate when disease severity is high, as in Australia. Otherwise, necrosis at the stem base can be monitored (Kaur et al. 2009; Jestin et al. 2011; Raman et al. 2020). The appearance of symptoms on the upper canopy of the crop was recently reported in some countries, and thus, included in the monitoring process, such as in Australia (Sprague et al. 2018; Raman et al. 2020). Molecular evaluation of the pathogenic DNA in the infected plant organ can be a parameter that assesses pathogen growth in diseased plants (Huang et al. 2014). The data collected from internal necrosis assessments were used for QTLs studies either directly or through different formula, such as that used to calculate the volume of diseased tissue (VDT) in the stem base or a disease index formula (Kutcher et al. 1993; Pilet et al. 1998; Aubertot et al. 2004).

This study aimed to compare the reliability of previously used inoculation methods to assess QDR with a method suggested by our group, which used mycelium as an inoculum applying it directly to the injured stem base. Another aim of this study was to phenotype an interconnected multiparent mapping (IMM) oilseed rape population for QDR against blackleg disease under controlled and natural conditions. The project dealt with plant materials from a wide genetic background as the population investigated was the result of crossing six parents that were reported to harbor a considerable level of QDR in the field with a susceptible parent.

# 2. Materials and methods

## 2.1 Screening *B. napus* DH lines for quantitative resistance against blackleg disease in the greenhouse

### 2.1.1 Plant material

A *B. napus* IMM population was produced by different oilseed rape breeding companies organized as members of the Department of Oil and Protein Crops in GFPi (Gemeinschaft zur Förderung von Pflanzeninnovation e. V.). The IMM population consists of 354 DH lines that were created after crossing the susceptible elite cultivar Lorenz with either of the six parental cultivars: A DH line from Jet Neuf (JN), Adriana, Alpaga, DK Cabernet, Galileo or King10, which are known to have QDR to *L. maculans* in the field (per. communication, NPZ Innovation GmbH, KWS SAAT Se and Syngenta Seeds GmbH, 2017). The subpopulations King10 x Lorenz, Adriana x Lorenz and JN x Lorenz were produced by NPZ Innovation GmbH (Holtsee, Germany) whereas the subpopulation Lorenz x Alpaga and Lorenz x DK Cabernet were produced by Syngenta Seeds GmbH (Bad Salzuflen, Germany), and the subpopulation Galileo x Lorenz by KWS SAAT Se (Einbeck, Germany) (Table 1). In total, 354 lines were screened in the greenhouse for QDR against blackleg disease.

**Table 1.** The parents of the interconnected multiparent mapping population, the breeding companies that produced the population, and the numbers of DH lines screened in the greenhouse and in the field.

| Parents with QDR | Susceptible parent | Breeding company | No. of DH lines screened | |
|---|---|---|---|---|
| | | | Field | Greenhouse |
| Galileo | Lorenz | KWS | 50 | 54 |
| King10 | Lorenz | NPZ | 50 | 60 |
| Adriana | Lorenz | NPZ | 50 | 60 |
| DH line Jet Neuf | Lorenz | NPZ | 50 | 60 |
| DK Cabernet | Lorenz | Syngenta | 50 | 60 |
| Alpaga | Lorenz | Syngenta | 50 | 60 |

### 2.1.2 Selection *of a L. maculans* isolate

The cornerstone of a successful assessment of QDR under controlled conditions is the right selection of both a reliable inoculation method and a suitable isolate of *L. maculans*. The selected isolate should not show a strong avirulance at cotyledon level when interacting with the tested plant material to allow for the unravelling of differences in quantitative resistance at stem level. To ensure this we tested the virulence of a collection of ten *L. maculans* isolates on cotyledons of the parent cultivars used to produce the IMM population. The isolates were

obtained from the *L. maculans* fungal collection established by a previous project in the Division of Plant Pathology and Crop Protection, Georg-August- University of Göttingen (Table 2).

**Table 2.** A collection of *L. maculans* isolates obtained from German fields. Isolate collection of the Division of Plant Pathology and Crop Protection, Georg-August-University of Göttingen.

| Isolate | Location | *B. napus* host | Year | *Virulence complexity | |
|---|---|---|---|---|---|
| | | | | *Rlm* | *LepR* |
| 1.1.1.55 | Grundhof | NK Bravour | 2013 | 1, 2, 3, 4, 9 | 2, 3 |
| 1.1.1.57 | Grundhof | NK Bravour | 2013 | 1, 2, 3, 4, 9 | 2, 3 |
| 1.1.1.66 | Grundhof | NK Bravour | 2013 | 1, 2, 3, 4, 9 | 2, 3 |
| 1.4.1.15 | Peine | NK Bravour | 2013 | 1, 2, 4, 7, 9 | 2, 3 |
| 1.4.1.65 | Peine | NK Bravour | 2013 | 1,2,3,4,7,9 | 2 |
| 2.2.1.28 | Malchow | NK Bravour | 2014 | 1, 2, 3, 4, 9 | 2, 3 |
| 2.2.1.29 | Malchow | NK Bravour | 2014 | 1, 2, 3, 4, 9 | 2, 3 |
| 2.4.1.29 | Peine | NK Bravour | 2014 | 1, 2, 4, 7, 9 | 2, 3 |
| 2.5.1.02 | Hadmersleben | NK Bravour | 2014 | 1, 2, 3, 4, 9 | 2, 3 |
| 2.7.1.06 | Thüle | NK Bravour | 2014 | 1, 2, 3, 4, 9 | 2, 3 |

*Virulence complexity was tested on a differential *B. napus* set that harbored resistance genes *Rlm*1-4, 7, 9 and *LepR*1-3.

For a cotyledon test, 10 µl of spore suspension ($10^7$ spores/ml) were applied on the cotyledon of the IMM population parents. Three replications were tested, and each cotyledon lobe was punctured with a sterile needle before applying the inoculum (3 replications x 4 lobes = 12 values). Plants were assessed 14 days post inoculation (dpi) using the IMASCORE scale, which includes six rating classes. Accordingly, classes one to three present incompatible reactions, where class one shows a hypersensitive reaction with a small lesion and class three shows a bigger lesion but without tissue collapse. Classes four to six represent compatible interactions, where plants of class four show tissue collapse but without sporulation as was the case with classes five and six (Balesdent et al. 2001). The experiment was conducted under temperatures of 20-23 °C and a 16/8 day/night system.

### 2.1.3 Selection of an inoculation method

The chosen isolate, which is coded as 1.4.1.15 (in short: isolate 15), was used to compare four different inoculation methods for the assessment of QDR in *B. napus* against blackleg disease. For this, the inoculation methods were tested on IMM population parent cultivars. Seeds were sown in a 1:3:3 mixture of sand, garden soil and compost in 11×11 cm pots. As

the plants reached the growth stage BBCH 13-14, when three to four true leaves unfolded (Lancashire et al. 1991), the inoculum was applied. Two types of inocula were used, either pycnidiospore suspensions or mycelial agar plugs. The inoculation was carried out by one of the following methods:

(1) spraying spore suspension ($10^7$ spores/ml) on the upper side of the first leaf

(2) spraying spore suspension ($10^7$ spores/ml) on the lower side of the first leaf

(3) applying a mycelial agar plug directly on an injured petiole of the first true leaf (one centimeter away from the stem)

(4) applying a mycelial agar plug on an injured stem base

Seven replications per treatment were assessed in a complete randomized design.

To prepare the mycelial agar plug inoculum, the spore suspension of isolate 15 was plated on V8-jucie medium supplied with 200 ppm streptomycin and incubated for 6-10 days under NUV light at 20°C. Five millimeter-plugs were then cut by a cork borer at the edge of the colonies, where the mycelia are more vital. A mycelial agar plug was put upside down at the center of an oatmeal agar medium (2% oatmeal, 1.5% agar) amended with 200 ppm of streptomycin. The plates were then incubated at room temperature in darkness for 10-14 days until the fungal colony was well grown and ready for the inoculation. To inoculate at the stem base, a superficial needle injury was made at the axil of the first true leaf and a mycelial-agar plug or an agar-plug was applied for control. To inoculate the petiole, a similar superficial injury was made before applying the inoculum one centimeter away from the stem axil.

After inoculation by any of the methods, plants were covered with a foil tunnel for 72 hours to develop a saturated humid condition for an optimal *L. maculans* infection. Plants were grown under controlled conditions at 20°C and under a 16/8 day/light system using Hortilux Schreder HPS 400W lamps (230V/50Hz). Plants were fertilized with Hakaphos Blau (Compo Expert, Germany) 0.6%. Symptoms were observed every two days to define the day of the first appearance of stem lesion. At 49 dpi, necrosis at stem bases was monitored. Lesion length (L), the girdling (G) of the stem and the diameter of the lesion profundity (P) were assessed after cross cutting the stem base. The values were given a category from one to nine based on a modified scale from Kutcher et al. (1993) (Table 3). The volume of diseased tissue (VDT) was then calculated according to a modified formula from Kutcher et al (1993):

$$VDT = \left(1 - \left(1 - \frac{P}{9}\right)^2\right) * \frac{G}{9} * L$$

The entire experiment was repeated in three rounds.

**Table 3.** A scale for assessment of a lesion caused by *Leptosphaeria maculans* on the stem base of *Brassica napus* modified from Kutcher et al. (1993).

| Length (mm) | Girdling (%) | Profundity (%) |
|---|---|---|
| 0=no infection | 0=no infection | 0=no infection |
| 1= 1-4 | 1=> 0-11 | 1=> 0-11 |
| 2= 5-8 | 2 = 12-22 | 2 = 12-22 |
| 3= 9-12 | 3 = 23-33 | 3 = 23-33 |
| 4= 13-16 | 4= 34-44 | 4= 34-44 |
| 5= 17-20 | 5= 45-55 | 5= 45-55 |
| 6= 21-24 | 6= 56-66 | 6= 56-66 |
| 7= 25-28 | 7= 67-77 | 7= 67-77 |
| 8= 29-32 | 8= 78-88 | 8= 78-88 |
| 0= > 32 | 9=> 88 | 9=> 88 |

### 2.1.4 Experimental design and disease assessment of IMM population

The DH lines of the IMM population were assessed in 11 rounds of greenhouse screenings. The phenotyping was conducted by inoculating a mycelial agar plug of isolate 15 at the stem base. Two replicates of each genotype were assessed per round. The experiment was carried out in a completely randomized design. At 14 dpi and 35 dpi disease assessment depended on measuring the length of the lesion on the stem base (mm) as well as a visual evaluation of the percentage of the girdling canker. At 49 dpi, besides the length (L) and the girdling (G) of the stem, the diameter of the lesion profundity (P) was assessed after cross cutting the stem base. The L, G and P values were categorized from one to nine according to the scale modified from Kutcher et al. (1993), shown in table 3.

Based on these scores, the total scoring value (TSV) was calculated as (L+G) per time point. The values of the TSV over time formed the disease progress curve and the area under the disease progress curve (AUDPC) was calculated according to the following formula (Madden et al., 2017):

$$\mathrm{AUDPC} = \sum_{j=1}^{n_j-1}\left(\frac{y_j + y_{j+1}}{2}\right)\left(t_{j+1} - t_j\right)$$

Where $t_j$ is the time point at which the measurements were taken, and the area under the infection curve was calculated based on the length and the girdling lesion on the stem base throughout the experimental period.

At 49 dpi, it was possible to crosscut the stem base at the lesion level and assess the profundity (P). VDT was then calculated according to a modified formula from Kutcher et al. (1993) described in 2.1.3.

## 2.2 Screening of quantitative resistance against blackleg disease in the field

### 2.2.1 Plant material

Three hundred DH lines from the IMM population described in (2.1.1) were assessed in the field. To enhance the infection, blackleg infested stubble collected from the same location at the end of the previous season was distributed in the field. Therefore, the interaction of the local natural races of *L. maculans* and plant material investigated here was also considered. Table 4 shows that the parental cultivars contained the major *R* genes *Rlm2*, *Rlm9*, *Rlm1* and *Rlm4*. According to Alnajar et al. (2022) (chapter II), *Rlm2* is completely ineffective in the regions where the field experiments were established. There were also indications from Alnajar et al. (2022) and Winter and Koopmann (2016) that *Rlm9* is not effective in Germany. The efficacy of *Rlm1* and *Rlm4* ranged from 3-19% and 3-13%, respectively (Table 5). Consequently, the major effect phenotyped in this plant material was related to QDR. NK Bravour was used as a trap susceptible control and Exocet was used as a reference cultivar that has the major *R* gene *Rlm7*, which was reported to be the most effective in German fields against blackleg disease in 2016 (Winter and Koopmann, 2016).

**Table 4.** Resistance background of the parental elite cultivars used to produce the interconnected multiparent population.

| Elite cultivars | Qualitative R | Quantitative R |
|---|---|---|
| Adriana | *Rlm2, Rlm4* | yes |
| JN NPZ DH line | *Rlm9, Rlm4* | yes |
| King 10 | *Rlm4* | yes |
| Galileo | *Rlm4* | yes |
| DK Cabernet | *Rlm1, Rlm2, Rlm9* | yes |
| Alpaga | *Rlm4* | yes |
| Lorenz | *Rlm9* | No |

Source: personal communication KWS SAAT Se (Einbeck, Germany).

**Table 5.** The efficacy of major *R* genes harbored in the parental cultivars of the interconnected multiparent mapping population produced for quantitative resistance screening under field conditions. The efficacy reflects the percentage of *L. maculans* isolates avirulent on the respected major *R* genes in a pathogenicity test according to Alnajar et al. (2022).

| Field trial locations | *Rlm1* | *Rlm2* | *Rlm4* | *Rlm9* |
|---|---|---|---|---|
| Hadmersleben | 14 | 0 | 6 | 0 |
| Groß Helle | 17 | 0 | 4 | 0 |
| Einbeck | 19 | 0 | 3 | 0 |
| Nienstädt | 16 | 0 | 12 | 0 |
| Peine | 3 | 0 | 13 | 0 |
| Asendorf | 8 | 0 | 13 | 0 |

### 2.2.2 Inoculation in the field and experimental design

Natural infection in the fields was augmented by adding infested oilseed rape stubble that showed *L. maculans* pseudothecia and were preserved from the previous season in each region for this goal. Infested stubble was chopped into small pieces. After sowing, 20 g of infested stubble was spread on each plot. Detailed characterizations of *L. maculans* races can be found in chapter II (in Alnajar et al. 2022).

Field experiments aimed to phenotype the IMM population under natural conditions. Trials were conducted over the three seasons 2017/2018, 2018/2019 and 2019/2020. In 2017/2018, field experiments were established in cooperation with breeding companies in four regions, namely Einbeck (KWS), Nienstädt (Monsanto), Groß Helle (NPZ) und Hadmersleben (Syngenta). In 2018/2019, similar experiments were conducted in Sörup (BASF), Hovedissen (Borries-Eckendorf), Leutewitz (DSV) and Peine (Limagrain). The third-season experiments (2019/2020) were implemented in Einbeck (KWS), Asendorf (DSV), Nienstädt (Bayer CropScience) and Peine (Limagrain).

Fifty DH lines from each subpopulation, the parents of the IMM population and two reference varieties, NK Bravour and Exocet were sown in the field between 28$^{th}$ August and 2$^{nd}$ September each season. The experiments had an alpha lattice design with two replications (Appendix 1). Plot size ranged from 3-6 m$^2$ and the seed density was 50 seeds/ m$^2$. Plant cultivation measures were performed according to good agricultural practices.

### 2.2.3 Disease assessment

Monitoring blackleg symptoms took place three times annually: in autumn at BBCH 18, in spring at BBCH 30-50 and in summer at BBCH 80-83. Monitoring in autumn and spring aimed to define the field with the highest disease pressure. Since phenotyping the entire IMM population is costly, we monitored it at BBCH 80-83 only in the field with the highest disease pressure. Monitoring in autumn and spring consisted of assessing 30 randomly selected plants per plot from 20 NK Bravour plots and 4 Exocet plots (720 plants). Disease incidence and disease severity was evaluated. Disease severity was assessed based on average number of lesions per plant in autumn. In spring, the incidence of the stem canker at the stem base was also considered. At the end of the season, 25 whole plant samples were collected from a selected location within the two replicates of each IMM population DH line (total: 350 lines X 2 replicates X 25 samples = 17500 plants). Samples were also taken from the parent plots and the reference varieties plots. VDT was calculated as described in (2.1.3).

### 2.3 Statistical analyses

Normal distribution of the VDT calculated from the greenhouse experiments and field monitoring values was checked. Afterwards, one-way ANOVA was applied using R software (R core Team 2021). When differences were significant, the post-hoc comparison was applied using Tukey test. Pearson´s correlation analysis was applied to reveal the relation between VDT and AUDPC.

## 3. Results

### 3.1 Phenotypic evaluation of IMM population under greenhouse conditions

To choose the right inoculum, the virulence of ten isolates was tested on cotyledons from seven parents of the IMM population. Table 6 showed that all isolates in our collection were virulent on all seven cultivars. However, isolates 1.4.1.65, 2.2.1.29, 1.2.1.15 and 2.5.1.02 were preferable as all resulting lesions showed grey-green tissue collapse and sporulation on the cotyledons i.e. they were rated 5 or 6 according to the IMASCORE scale (class 4 had lesions without sporulation). However, isolate 1.2.1.15 was chosen for further greenhouse assessment as it showed better viability by growing on the V8 culture medium - we refer to it as isolate 15 in our study.

**Table 6.** Virulence test of *L. maculans* isolates on cotyledons of the IMM population parents for the proof of absence of gene-for-gene interactions. Interactions were scored from 1 to 6 according to the IMASCORE scale. Values are the average of 12 scores (3 replication X 4 lobes).

| Parents of the IMM population | *L.maculans* isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2.5.1.02 | 2.7.1.06 | 1.1.1.55 | 1.2.1.15 | 2.2.1.28 | 2.2.1.29 | 2.4.1.29 | 1.1.1.57 | 1.4.1.65 | 1.1.1.66 |
| Lorenz | 6 | 6 | 6 | 5 | 5 | 5 | 5 | 6 | 6 | 6 |
| Adriana | 5 | 5 | 5 | 6 | 4 | 5 | 4 | 5 | 5 | 4 |
| Jet Neuf x DH Linie NPZ | 6 | 6 | 6 | 6 | 5 | 6 | 6 | 6 | 6 | 6 |
| King 10 | 5 | 5 | 4 | 5 | 5 | 5 | 4 | 4 | 5 | 4 |
| Galileo | 5 | 4 | 5 | 6 | 4 | 6 | 5 | 5 | 5 | 5 |
| Alpaga | 6 | 5 | 5 | 6 | 5 | 6 | 6 | 6 | 6 | 6 |
| DK Cabernet | 5 | 5 | 5 | 6 | 5 | 5 | 5 | 5 | 5 | 5 |

Isolate 15 was used to compare the potential of different inoculation methods to reliably differentiate QDR against blackleg disease among *B. napus* DH lines. Figure 1 showed that the average VDT at the stem base of the susceptible cultivar Lorenz was 5.7 when a mycelial agar plug was inoculated directly on an injured stem showing higher susceptibility to blackleg than other parents of the IMM population (Figure 1, G and H). That is in alignment with reports of the breeding companies who produced the IMM population. These observed that Lorenz was susceptible to blackleg in the field while the other six parents presented different levels of QDR. In contrast, VDT values on the stems of Lorenz were as low as 1.3 when spraying the first leaf with spore suspension on its upper side (Figure 1, E and F) and ca. 3 when applying spore suspension on its underside, or by inoculating its injured petiole (Figure 1, A, B, C and D). Inoculation with a mycelial agar plug on the petiole or on the stem showed a similar tendency in all three repetitions for the entire experiment, where Lorenz was the most susceptible variety although its VDT value differed slightly from experiment to experiment. On the contrary, inoculating the upper or undersides of the leaves led to fluctuating results compared with inoculation at the stem base or petiole. It was observed that the inoculated leaf or the leaf whose petiole was inoculated dropped down after 10-14 dpi. This might affect the inoculum load that was supposed to arrive at the stem through the petiole. Consequently, disease severity varied at the stem base when last monitored, i.e. 49 dpi. However, 95% of plants whose petiole was inoculated showed lesions on the stem as early as 7 dpi, whereas 28% of the plants whose leaves were infected on the lamina fell before a phoma lesion

appeared on the stem. We concluded that inoculation with a mycelial agar plug on stem base is relatively the most reliable and reproducible inoculation method found through this study, followed by inoculation on petiole. Inoculation on the lamina seems to be least reliable with no difference between inoculation on the upper or under side.

Eleven screening rounds were conducted to evaluate the DH lines of the IMM population using a mycelial agar plug on the injured stem base in the greenhouse. Results were pooled to give 22 replications per DH line. VDT values showed distinct differentiation between the DH lines in QDR for a *L. maculans* infection among the IMM population (Figure 2). These were further used to define QTLs involved in QDR (chapter IV). The DH lines showed variation among the IMM population as they were rated by the AUDPC formula as well (Figure 3). Correlation analysis showed a significantly positive correlation between AUDPC and VDT ($R^2$= 0.69 at P= 0.01) (Figure 4). Both rating formulae can be used to phenotype oilseed rape accessions for QDR against blackleg. However, the results of our study showed more potential of VDT to unravel relevant differences between the DH lines investigated. AUDPC rating formula has the advantage of considering the temporal changes of the lesion. However, it does not differentiate two DH lines that differ in lesion profundity as this parameter is excluded in the AUDPC formula. Through our monitoring work, we observed that cutting the stem and evaluating the lesion inside it is indispensable to objectively depict blackleg disease severity. We noticed that the pathogen could grow in two different forms when the infection started from a point injury directly at the stem base. In its first form, the pathogen could spread at the epidermis, in the cortex and in the pith in a parallel structure. In this case, the outer length of the lesion at the epidermis was equal to the inner length inside the stem (Figure 5, A). The second form showed that the pathogen was able to grow from the infection point directly to the cortex without being widespread in the epidermis. However, it still grew to the pith (Figure 5, B). Thus, the outer length of the lesion was smaller than the inside length of the lesion. Until now there is no non-destructive method that enables us to monitor the development of the lesion inside the stem over time.

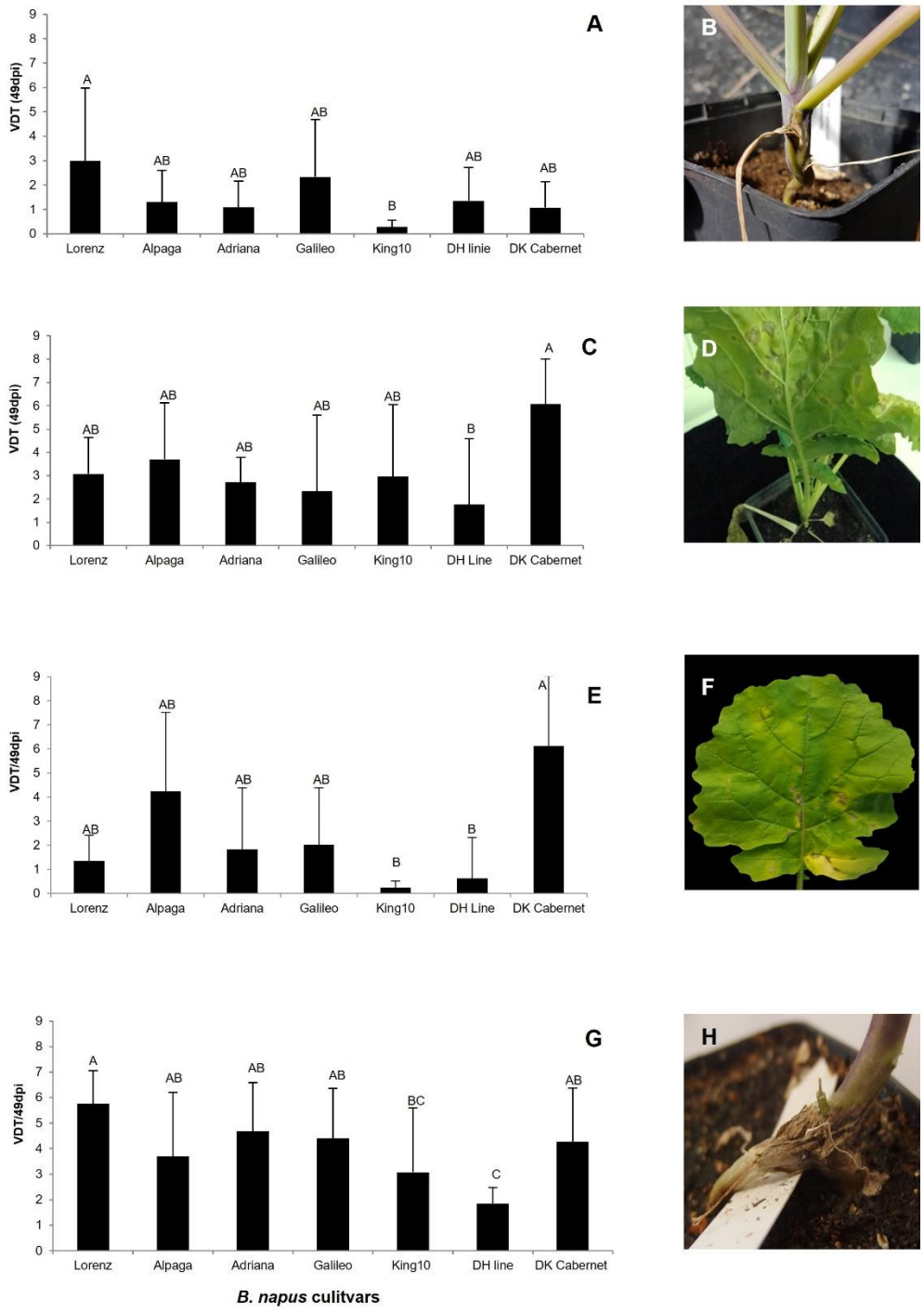



**Figure 1.** Evaluation of quantitative resistance against *L. maculans* at the stem base of seven *B. napus* cultivars using four different inoculation methods. **A, B:** Inoculation with mycelial agar plugs on injured petioles. **C, D:** Inoculation by spraying spore suspension on the leaf´s underside. **E, F:** Inoculation with spore suspension on the leaf´s upper side. **G, H:** inoculation with a mycelial agar plug on an injured stem base. Volume of the diseased tissue (VDT) was calculated at 49 dpi. Letters show significant differences according to the Kruskal-Wallis Test followed by post hoc comparisons with the Dunn procedure (P< 0.05). Photos show symptoms on the susceptible cultivar Lorenz.

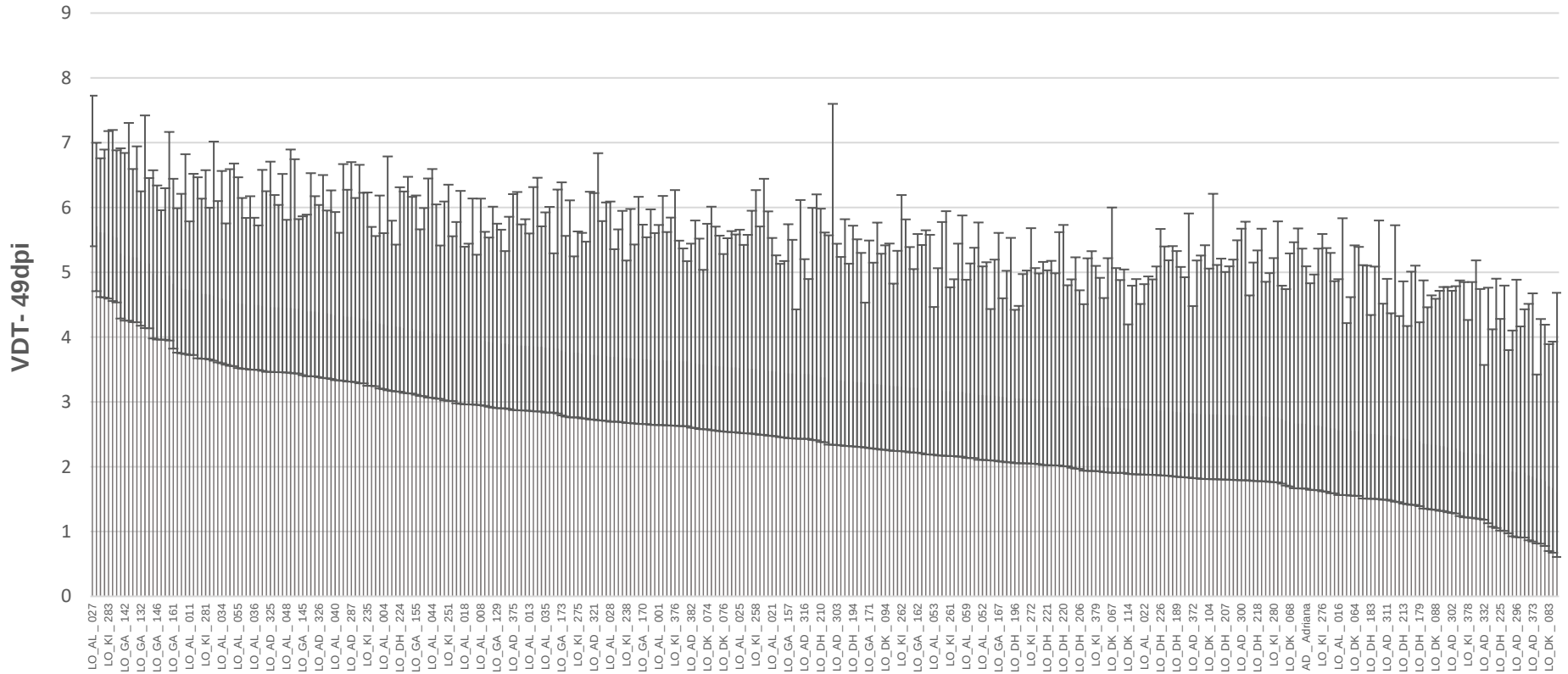



**Figure 2.** Assessment of blackleg quantitative resistance of 354 DH lines of *B. napus* interconnected multiparent mapping population. Bars represent the average disease severity of 22 replications based on evaluating the volume of diseased tissues (VDT) at the stem base at 49 dpi. LO: Lorenz, AD: Adriana, DK: DK Cabernet, KI: King10, DH: DH line from Jet Neuf (JN), GA: Galileo, AL: Alpaga.

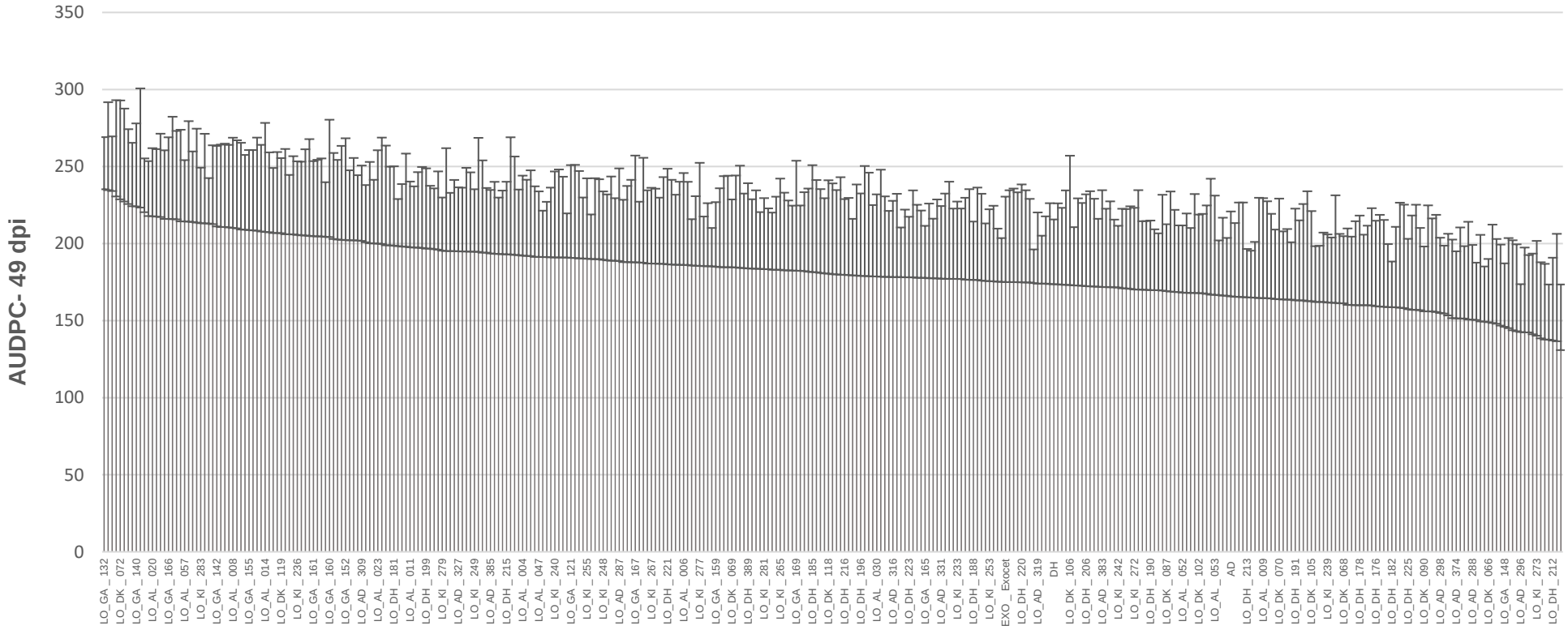



**Figure 3.** Assessment of blackleg quantitative resistance of 354 DH lines of *B. napus* interconnected multiparent mapping population. Bars represent the average disease severity of 22 replications based on evaluating the area under disease progress curve (AUDPC) at 49 dpi. LO: Lorenz, AD: Adriana, DK: DK Cabernet, KI: King10, DH: DH line from Jet Neuf (JN), GA: Galileo, AL: Alpaga.

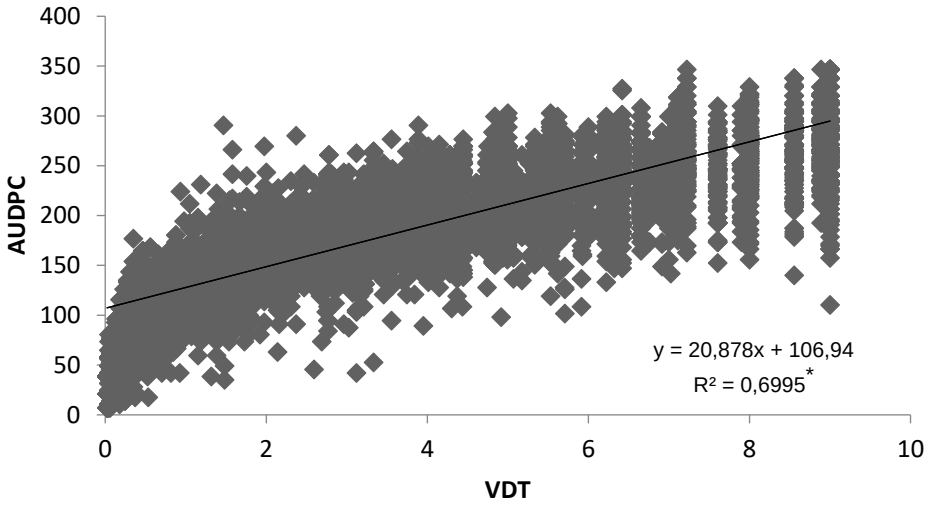



**Figure 4.** Correlation of the volume of diseased tissue (VDT) at the stem base of oilseed rape - the area under the diseased progress curve (AUDPC) of plants infected with *L. maculans.* Pearson correlation showed significant correlation at P=0.05.

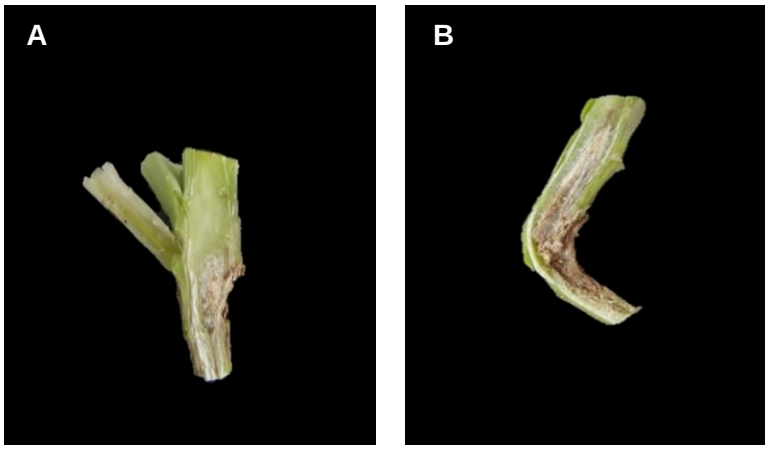



**Figure 5.** Different forms of blackleg canker at and in stem bases of oilseed rape infected plants. (A) Length of cankered epidermis is equal to the inside length of the internal canker in the vascular system. (B) Outer length of the lesion is smaller than the longitude growth inside the cortex and the pith.

### 3.2 Phenotypic evaluation of IMM population in the field

The IMM population was evaluated for QDR against blackleg in several regions over three seasons. Four field experiments were established every season. Due to the labour intensity and the high cost required for phenotyping the whole IMM population, sampling to monitor stem canker in the DH lines was conducted in the field that showed the highest disease pressure. The highest disease pressure was defined based on monitoring Phoma symptoms

on the susceptible cultivar NK Bravour in autumn and spring. Phenotyping results in autumn indicated that disease pressure was the highest in Nienstädt in 2017/2018 and in Peine in 2019/2020 with a disease incidence of 99.8% and 100%, and disease severity of 92.2 and 50,8 leaf spots/plants, respectively (Table 7). However, in the 2018/2019 season, all plants in the four field trials (Sörup, Leutewitz, Peine and Hovedissen) showed virtually no phoma symptoms due to high temperatures and drought at the trial locations during the growing season. After the establishment of the disease through the primary infection in autumn, the pathogen grew symptomless in the stem but turned necrotrophic once it reached the stem base. Thus, the canker formation at the plant-stem base was only recognised at the beginning of spring. Therefore, by monitoring in spring, disease pressure was defined additionally based on the occurrence of a primary stem canker at the stem base, regardless of the canker profundity which was not expected to be deep within the stem at that growth stage.

**Table 7.** *L. maculans* disease incidence and disease severity in field trials evaluated in the 20 plots of the trap variety NK Bravour at the BBCH18 and BBCH30-50 in different regions in Germany, DI= disease incidence, DS= disease severity.

| Season | Regions | BBCH18 | | BBCH30-50 | | |
|---|---|---|---|---|---|---|
| | | DI (%) | DS-leaf spot | DI (%) | DS leaf spots/plant | stem canker (%) |
| 2017/2018 | Hadmersleben | 90,05 | 8,08 | 86.00 | 9.00 | 1.00 |
| | Einbeck | 96,45 | 10,61 | 93.18 | 26.92 | 0.05 |
| | Groß Helle | 97,50 | 14,90 | 18.09 | 1.54 | 0.00 |
| | Nienstädt | 99,80 | 92,20 | 98.00 | 18.00 | 51.00 |
| 2018/2019 | Sörup | 1 | 1 | 1 | 2 | 0 |
| | Peine | 2 | 1 | 8 | 2 | 0 |
| | Leutewitz | 0 | 0 | 0 | 0 | 0 |
| | Hovedissen | 0 | 0 | 0 | 0 | 0 |
| 2019/2020 | Einbeck | 98,2 | 8,2 | 98,67 | 53,21 | 0,67 |
| | Asendorf | 97,42 | 22,18 | 100 | 19,47 | 21,57 |
| | Nienstädt | 95,51 | 28,62 | 79,17 | 3,49 | 0,8 |
| | Peine | 100 | 50,03 | 94,67 | 12,74 | 33 |

In season 2017/2018, disease incidence in spring was very close in both Einbeck and Nienstädt. Samples from Einbeck had the highest average number of phome leaf spots (26.92 leaf spots/plant). However, Nienstädt had the highest stem canker incidence (51%) (Table 7). Since the latter is economically the more relevant parameter at the end of the season, the IMM

population was monitored in Nienstädt in the respective season. In season 2019/2020, disease incidence was the highest in Peine with 100% of the plants having an average of 50.03 phoma leaf spots/plant. High incidence of stem canker in spring was registered in Peine (33%) as well. However, a block of the two replications in Peine was destroyed due wild boars raiding the crop. Therefore, Asendorf was chosen for the final monitoring of the IMM population. There, 100% of the plants had phoma leaf symptoms in spring with 19.47 spots/plant on average - 21.57% of the plants showed preliminary stem canker at the stem base (Table 7).

Phenotyping of the DH lines at the BBCH80-83 showed significant variation among the 300 DH lines with VDT values ranging from 2.5 to 8.2 in season 2017/2018 (Figure 8A, Appendix 2). As expected for quantitative traits, the VDT values were normally distributed, which confirmed the quantitative character of this type of resistance (Figure 6A). Surprisingly, VDT values in season 2019/2020 were not normally distributed (Figure 6B), and the average value of VDT for the DH lines ranged between 0.07 and 1.7 (Figure 8B). Although plants showed clear Phoma lesions on both the upper stem and the stem base (Figure 7A), VDT showed very low values. VDT values were influenced by severe damage mainly caused by the larvae of the rape stem weevil (*Ceutorhynchus napi*) which feeds on the pith and tunnels within the stem (Figure 7B). The weevil damage was so severe that it was not possible to distinguish the grade of fungus damage, and therefore the level of plant resistance. Insect damage occurred despite the application of neonicotinoid systemic insecticide Biscaya in a concentration of 0.3 L/ha. The insecticide was sprayed three times per season: in autumn 07.10.2019, and in spring 09.04.2020 and 24.04.2020. Results showed that 100% of the plants were damaged by insects, mainly by the rape stem weevil larvae. This can be attributed either to a strong neonicotinoid resistance in Asendorf or incorrect timing of insecticide application. Overall, the season 2019/2020 emphasized the central role of other biotic factors for a successful phenotyping for quantitative resistance in the field, such as wild boar or insect damage.

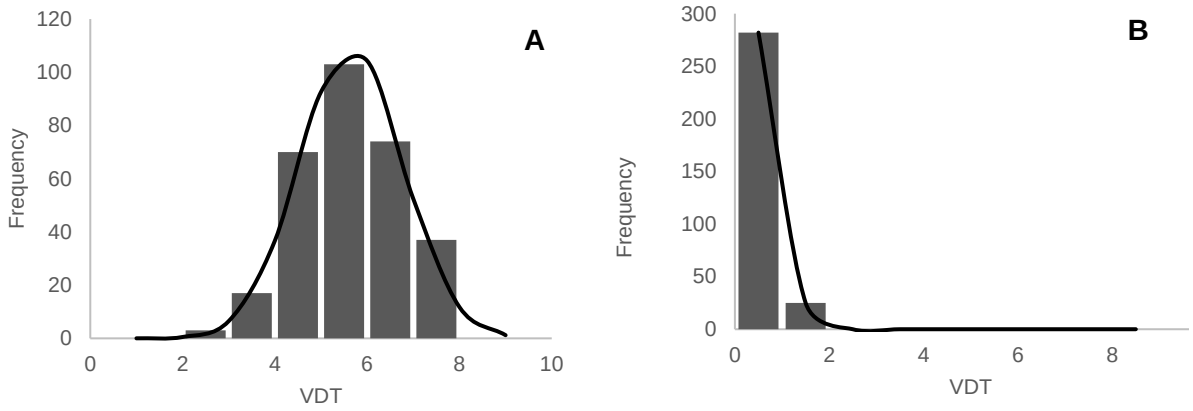



**Figure 6.** Distribution of the volume of diseased tissues (VDT). Mean values recorded by evaluating quantitative resistance against *L. maculans* in oilseed rape DH lines produced from an interconnected multiparent population in (A) Nienstädt, and in (B) Asendorf.


**Figure 7.** Damage of oilseed rape stems caused by biotic factors: (A) Phoma stem lesions on samples taken from Asendorf, a field in which phenotyping for quantitative resistance against *L. maculans* was conducted; (B) Damage inside the stem caused by the larvae of the rape stem weevil.

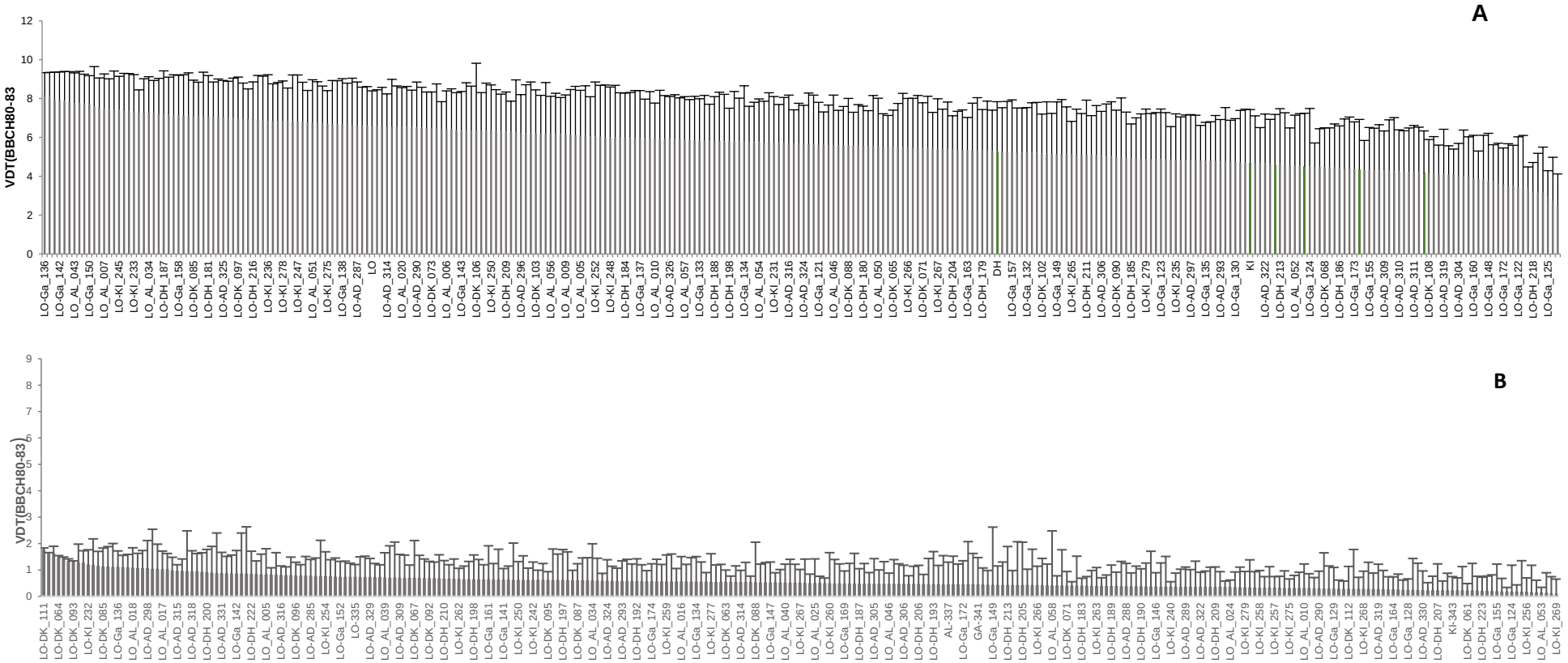



**Figure 8.** Assessment of blackleg quantitative resistance of DH lines produced from interconnected multiparent mapping population of *B. napus* in the field. A: in season 2017/2018, and B: in season 2019/2020. Bars represent the average disease severity of 25 samples X 2 replications based on evaluating the volume of diseased tissues (VDT) at the stem base at BBCH80-83.

## 4. Discussion

The phenotypic evaluation conducted in this study showed that the DH lines of the IMM population distinctly differed in QDR to the *L. maculans* infection. This variation was the basis for the genome-wide association study carried out at the Justus-Liebig University of Gießen, Germany to identify genomic regions that contributed to QDR (Vollrath et al. 2021). Vollrath et al. (2021) defined eight significantly associated QTLs with small effects that explained 3-6% of the variation. These QTLs are located on chromosomes A07 and A09 in *B. napus* (see chapter IV).

It is known that in order to phenotype plants for QDR, it is important to allow the pathogen further growth beyond the barriers made by major *R* gene mediated resistance at the cotyledon stage (Huang et al. 2014). All isolates tested with the parental varieties of the IMM population in this study showed compatible reactions on the cotyledons. This meant that the isolates neither have the avirulence genes *Avrlm1*, *Avrlm2*, *Avrlm4* and *Avrlm9* that correspond to the major *R* genes described in the IMM population, nor do they have any other avirulence genes that might interact with other unknown major *R* genes in the genotypes. This conclusion depends on the gene-for-gene concept from Flor (1971).

In this study we compared different inoculation methods that used various fungal inocula (pycnidiospores or mycelium) for a range of plant organs. We showed that using the *L. maculans* mycelial agar plug as an inoculum and applying it directly on an injured stem base is the most reliable inoculation method. Other inoculation methods used different types of inocula such as ascospores (Huang et al. 2014). The advantage of ascospores is that they germinate two days faster than pycnidiospores (Li et al. 2004) and cause more severe infections (Li et al. 2006). However, by using ascospores it is difficult to choose the isolates and this method depends on using the natural population of the region from which the stubble was sampled (Huang et al. 2014). In this study, we showed that mycelium can be used successfully as an inoculum. In fungi, the mycelium function is mainly to allow the microorganism to expand and direct it towards sources of nutrient acquisition or mating (Brand and Gow, 2009). Although mycelium plays a role in invading plant tissue and evasion resistance barriers, it is not the main initiator of the infection (Gow et al. 2002). Through providing the point of injury as an entry point for the mycelium, the hemibiotroph *L. maculans* could act immediately as a necrotrophic pathogen. This resulted in a similar canker at the stem base as the canker occurred when the infection starts at the lamina or petiole.

A variation in QDR against blackleg disease in oilseed rape can be seen only when the symptoms appear on stem level and not during the symptomless phase (Huang et al. 2009). Huang et al. (2009) compared the symptomless phase of *L. maculans* in a genotype with QDR

and another without as the fungus grew from the leaf lamia to the stem after inoculating the plants with an ascospore shower under controlled conditions. Results showed that the incubation period (symptomless phase) did not differ significantly between the two genotypes. This was confirmed by qPCR and microscopically by observing GFP-expressing *L. maculans*. Despite this, stem canker severity was significantly different between the genotypes (Huang et al. 2009, 2014). Thus, it can be assumed that using an agar plug directly at the stem can reflect the QDR differences objectively. This method allowed us to avoid the dropping of the leaves before the fungus reached the stem. Out of the eight QTLs detected in the IMM population, using the on-stem agar plug technique, three were detected in a field phenotyping experiment (Vollrath et al. 2021, chapter IV). Raman et al. (2018) detected 31 QTLs using the ascospore shower inoculation method, of which nine were consistently detected- only two were common QTLs within the field experiments.

The selection of a suitable assessment approach is pivotal for high throughput results in QTL studies. In our study, we used both VDT and AUDPC to assess symptoms from greenhouse phenotyping, and only the destructive VDT for assessment of field samples at BBCH 80-83. Van den Berg et al. (1993) emphasized that the assessment approach should consider the uttermost symptom expansion. Aubertot et al. (2004) compared different methods to assess blackleg disease in field samples and showed that assessing cross-sections at the stem base was more reliable compared to the visual assessment of the outer lesion alone without cutting the stem. In this context, it is important to match the approach with the scientific question of the study. For instance, while AUDPC is suitable for studies that consider temporal changes of symptoms and is a non-destructive method, VDT is more appropriate when assessment of lesion depth at the end of the experiment in a cross-section is important. Van den Berg et al. (1993) stated that depending on external symptoms can lead to an over- or underestimation of disease severity. This was also illustrated in our study by the types of the internal infection described in the greenhouse (Figure 5).

In general, QTLs can be detected based on data genetrated under controlled conditions (Raman et al. 2016), or data produced in field experiments (Jestin et al. 2015) or both (Raman et al. 2018). However, the detection of common QTLs in both the field and the greenhouse reflect the genotype × environment interaction as phenotypic variance in quantitative traits are the results of the related genetic regions (QTLs), their interactions with one another and their interaction with the environment (Powder, 2020). In our study, we established four field experiments per year. However, we chose one field for final monitoring based on phoma symptom assessments in autumn and spring to reduce the costs. Since correlation between phoma leaf lesions and cankers at the stem base level is very poor (Huang et al. 2009), we consider the leaf lesions only as an indicator. Nevertheless, we depended on the preliminary

appearance of stem canker in spring to speculate about the pressure of blackleg under field conditions for IMM phenotyping.

The IMM population showed significant variation in the field in the first season. However, biotic and abiotic stresses were serious constraints for phenotyping in the following seasons. In 2018, our field experiments showed no phoma leaf lesions in autumn and consequently no stem canker in summer 2019. Expert reports documented a prolonged heat and dryness wave in Europe in 2018 (EDO analytical report, 2018). This could explain the lack of symptoms since leaf wetness duration is a crucial factor for *L. maculans* infections. Under sub-optimal infection criteria, such as drought, the infection efficacy of ascospores decreases (Biddulph et al. 1999). Among the biotic challenges was the crop damage by wild boars. Schlageter (2015) showed that this is an increasingly important challenge in Europe that needs to be controlled. In this context, acoustic signals, lasers or electric fences were suggested for crop protection (Schlageter 2015).

Another biotic stress that affected our results was the insect damage. Phenotyping results to assess stem damage caused by *L. maculans* gave biased results due to insects that tunnelled the stems. The failure to control insect damage in the third season can be possibly explained by choosing the wrong timing for insecticide application or by neonicotinoid resistance (Biscaya). This shows that the drawback of insecticide resistance is a problem not only for agricultural production, but also for scientific research. Initial reports on neonicotinoid resistance were published in 1996. Since then, a drastic increase in arthropod species resistance to neonicotinoids have been documented (Bass et al. 2015).

To sum up, this study suggested that applying a mycelial agar plug directly on the stem base of oilseed rape can be an effective inoculation method for phenotyping under controlled conditions. This method was successfully used to phenotype a large mapping population and to detect new QTLs involved in QDR. A reliable phenotyping method under greenhouse conditions can accelerate the process of searching for new sources of QDR with lower costs. However, phenotyping in the field is indespensible for the commercialized use of the markers detected. We described practical problems that need to be managed as prerequsites for successful phenotyping of QDR in the field.

## 5. Acknowledgement

The authors of this chapter thank the German Federal Ministry for Food and Agriculture (BMEL) for funding this project. We also acknowledge the following colleagues for supporting the sampling and assessment of field samples: Jutta Schaper, Heike Rollwage, Isabel Müller, Evelin Vorbeck, Maik Knobel and Hubertus Reintke, Al-Mamun MD, Aleem Ahmed, Balint

Bakondi, Yazan Taher and Maria Belen Ledesma. We thank Jutta Schaper and Heike Rollwage for their technical assistance in the phenotypic assessment in the greenhouse.

## 6. References

Amas, J., Anderson, R., Edwards, D., Cowling, W., and Batley, J. 2021. Status and advances in mining for blackleg (*Leptosphaeria maculans*) quantitative resistance (QR) in oilseed rape (*Brassica napus*). *Theor. Appl. Genet.* 134: 3123–3145. doi:10.1007/s00122-021-03877-0.

Aubertot, J.-N., Schott, J.-J., Penaud, A., Brun, H., and Doré, T. 2004. Methods for sampling and assessment in relation to the spatial pattern of phoma stem canker (*Leptosphaeria maculans*) in oilseed rape. *Eur. J. of Plant Pathol.* 110: 183–192. doi:10.1023/B:EJPP.0000015359.61910.3b.

Balesdent, M.H., Attard, A., Ansan-Melayah, D., Delourme, R., Renard, M., and Rouxel, T. 2001. Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans*. *Phytopathol.* 91: 70–76. doi:10.1094/PHYTO.2001.91.1.70.

Bass, C., Denholm, I., Williamson, M.S., and Nauen, R. 2015. The global status of insect resistance to neonicotinoid insecticides. *Pestic. Biochem. Phys.* 121: 78–87. doi:10.1016/j.pestbp.2015.04.004.

van den Berg, C.G.J., Rimmer, S.R., and Parks, P. 1993. Comparison of scales for rating severity of blackleg in canola. *Can. J. Plant Pathol.* 15: 49–53. doi:10.1080/07060669309500851.

Biddulph, J.E., Fitt, B.D.L., Leech, P.K., Welham, S.J., and Gladders, P. 1999. Effects of temperature and wetness duration on infection of oilseed rape leaves by ascospores of *Leptosphaeria maculans* (stem canker). *Eur. J. Plant Pathol.* 105: 769–781. doi: https://doi.org/10.1023/A:1008727530088

Brand, A., and Gow, N.A. 2009. Mechanisms of hypha orientation of fungi. *Curr. Opin. Microbiol.* 12: 350–357. doi:10.1016/j.mib.2009.05.007.

Brun, H., Chèvre, A., Fitt, B.D., Powers, S., Besnard, A., Ermel, M., Huteau, V., Marquer, B., Eber, F., Renard, M., and Andrivon, D. 2010. Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytol.* 185: 285–299. doi:10.1111/j.1469-8137.2009.03049.x.

Cobb, J.N., DeClerck, G., Greenberg, A., Clark, R., and McCouch, S. 2013. Next-generation phenotyping: requirements and strategies for enhancing our understanding of genotype–phenotype relationships and its relevance to crop improvement. *Theor. Appl. Genet.* 126: 867–887. doi:10.1007/s00122-013-2066-0.

Delourme, R., Bousset, L., Ermel, M., Duffé, P., Besnard, A.L., Marquer, B., Fudal, I., Linglin, J., Chadœuf, J., and Brun, H. 2014. Quantitative resistance affects the speed of frequency increase but not the diversity of the virulence alleles overcoming a major resistance gene to *Leptosphaeria maculans* in oilseed rape. *Infect. Genet. Evolut.* 27: 490–499. doi:10.1016/j.meegid.2013.12.019.

EDO analytical report 2018. Drought in central-northern Europe – September 2018. URL: https://edo.jrc.ec.europa.eu/documents/news/EDODroughtNews201809_Central_North_Europe.pdf. Accessed on: 04. 11. 2022

Elferjani, R., and Soolanayakanahally, R. 2018. Canola responses to drought, heat, and combined stress: shared and specific effects on carbon assimilation, seed yield, and oil composition. *Front. Plant Sci.* 9: 1224. doi:10.3389/fpls.2018.01224.

Fikere, M., Barbulescu, D.M., Malmberg, M.M., Maharjan, P., Salisbury, P.A., Kant, S., Panozzo, J., Norton, S., Spangenberg, G.C., Cogan, N.O.I., and Daetwyler, H.D. 2020. Genomic prediction and genetic correlation of agronomic, blackleg disease, and seed quality traits in canola (*Brassica napus* L.). *Plants* 9: 719. doi:10.3390/plants9060719.

Flor, H.H. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9: 275–296. doi:10.1146/annurev.py.09.090171.001423.

Fortune, J.A., Qi, A., Ritchie, F., Karandeni Dewage, C.S., Fitt, B.D.L., and Huang, Y. 2021. Effects of cultivar resistance and fungicide application on stem canker of oilseed rape (*Brassica napus*) and potential interseasonal transmission of *Leptosphaeria* spp. inoculum. *Plant Pathol.* 70: 2115–2124. doi:10.1111/ppa.13453.

Gow, N.A.R., Brown, A.J.P., and Odds, F.C. 2002. Fungal morphogenesis and host invasion. *Curr. Opin. Microbiol.* 5: 366–371. doi:10.1016/S1369-5274(02)00338-7.

Gupta, P.K., Kulwal, P.L., and Jaiswal, V. 2019. Association mapping in plants in the post-GWAS genomics era. *Adv. Genet.* 104: 75–154. doi:10.1016/bs.adgen.2018.12.001.

Huang, Y.J., Jestin, C., Welham, S.J., King, G.J., Manzanares-Dauleux, M.J., Fitt, B.D.L., and Delourme, R. 2016. Identification of environmentally stable QTL for resistance against *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *Theor. Appl. Genet.* 129: 169–180. doi:10.1007/s00122-015-2620-z.

Huang, Y.-J., Paillard, S., Kumar, V., King, G.J., Fitt, B.D.L., and Delourme, R. 2019. Oilseed rape (*Brassica napus*) resistance to growth of *Leptosphaeria maculans* in leaves of young plants contributes to quantitative resistance in stems of adult plants. *PLoS ONE* 14: e0222540. doi:10.1371/journal.pone.0222540.

Huang, Y.J., Pirie, E.J., Evans, N., Delourme, R., King, G.J., and Fitt, B.D.L. 2009. Quantitative resistance to symptomless growth of *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape). *Plant Pathol.* 58: 314–323. doi:10.1111/j.1365-3059.2008.01957.x.

Huang, Y.-J., Qi, A., King, G.J., and Fitt, B.D.L. 2014. Assessing quantitative resistance against *Leptosphaeria maculans* (Phoma stem canker) in *Brassica napus* (oilseed rape) in young plants. *PLoS ONE* 9: e84924. doi:10.1371/journal.pone.0084924.

Jestin, C., Bardol, N., Lodé, M., Duffé, P., Domin, C., Vallée, P., Mangin, B., Manzanares-Dauleux, M.J., and Delourme, R. 2015. Connected populations for detecting quantitative resistance factors to phoma stem canker in oilseed rape (*Brassica napus* L.). *Mol. Breed.* 35: 167. doi:10.1007/s11032-015-0356-8.

Jestin, C., Lodé, M., Vallée, P., Domin, C., Falentin, C., Horvais, R., Coedel, S., Manzanares-Dauleux, M.J., and Delourme, R. 2011. Association mapping of quantitative resistance for *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). *Mol Breed.* 27: 271–287. doi:10.1007/s11032-010-9429-x.

Kaur, S., Cogan, N.O.I., Ye, G., Baillie, R.C., Hand, M.L., Ling, A.E., Mcgearey, A.K., Kaur, J., Hopkins, C.J., Todorovic, M., Mountford, H., Edwards, D., Batley, J., Burton, W., Salisbury, P., Gororo, N., Marcroft, S., Kearney, G., Smith, K.F., Forster, J.W., and Spangenberg, G.C. 2009. Genetic map construction and QTL mapping of resistance to blackleg (*Leptosphaeria maculans*) disease in Australian canola (*Brassica napus* L.) cultivars. *Theor. Appl. Genet.* 120: 71–83. doi:10.1007/s00122-009-1160-9.

Kutcher, H.R., van den Berg, C.G.J., and Rimmer, S.R. 1993. Variation in pathogenicity of *Leptosphaeria maculans* on *Brassica* spp. based on cotyledon and stem reactions. *Can. J. Plant Pathol.* 15: 253–258. doi:10.1080/07060669309501920.

Lancashire, P.D., Bleiholder, H., Boom, T.V.D., Langelüddeke, P., Stauss, R., Weber, E., and Witzenberger, A. 1991. A uniform decimal code for growth stages of crops and weeds. *Ann. Appl. Biol.* 119: 561–601. doi:10.1111/j.1744-7348.1991.tb04895.x.

Li, H., Sivasithamparam, K., Barbetti, M.J., and Kuo, J. 2004. Germination and invasion by ascospores and pycnidiospores of *Leptosphaeria maculans* on spring-type *Brassica napus* canola varieties with varying susceptibility to blackleg. *J. Gen. Plant Pathol.* 70: 261–269. doi:10.1007/s10327-004-0125-8.

Li, H., Tapper, N., Dean, N., Barbetti, M., and Sivasithamparam, K. 2006. Enhanced pathogenicity of *Leptosphaeria maculans* pycnidiospores from paired co-inoculation of *Brassica napus* cotyledons with ascospores. *Ann. Bot.* 97: 1151–1156. doi:10.1093/aob/mcl062.

Madden, L.V., Hughes, G., and van den Bosch, F. 2017. Temporal analysis I: quantifying and comparing epidemics. In: Madden, L.V., Hughes, G., and van den Bosch, 2017. The study of plant disease epidemics, APS, 63-116. doi:10.1094/9780890545058. ISBN:978-0-89054-505-8.

Marcroft, S.J., Elliott, V.L., Cozijnsen, A.J., Salisbury, P.A., Howlett, B.J., and Van de Wouw, A.P. 2012. Identifying resistance genes to *Leptosphaeria maculans* in Australian

*Brassica napus* cultivars based on reactions to isolates with known avirulence genotypes. *Crop Pasture Sci.* 63: 338. doi:10.1071/CP11341.

McDonald, B.A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* 40: 349–379. doi:10.1146/annurev.phyto.40.120501.101443.

Nickel, R. 2021. Canada's drought forces canola importers to turn elsewhere. Reuters. URL: https://www.reuters.com/article/canada-canola-idUSKBN2GU0ZI. Accessed on: 09. 03. 2022

Obermeier, C., and Friedt, W. 2015. Applied oilseed rape marker technology and genomics, 253–295. *In:* Poltronieri, P. and Hong, Y. 2015. Applied plant genomics and biotechnology. Elsevier. doi:10.1016/B978-0-08-100068-7.00016-1. ISBN: 978-0-08-100068-7

Parlevliet, J.E. 2002. Durability of resistance against fungal, bacterial and viral pathogens; present situation. *Euphytica* 124: 147–156. doi:10.1023/A:1015601731446.

Pilet, M.L., Delourme, R., Foisset, N., and Renard, M. 1998. Identification of QTL involved in field resistance to light leaf spot (*Pyrenopeziza brassicae*) and blackleg resistance (*Leptosphaeria maculans*) in winter rapeseed (*Brassica napus* L.): *Theor. Appl. Genet.* 97: 398–406. doi:10.1007/s001220050909.

Pink, D., Bailey, L., McClement, S., Hand, P., Mathas, E., Buchanan-Wollaston, V., Astley, D., King, G., and Teakle, G. 2008. Double haploids, markers and QTL analysis in vegetable brassicas. *Euphytica* 164: 509–514. doi:10.1007/s10681-008-9742-1.

Poland, J.A., Balint-Kurti, P.J., Wisser, R.J., Pratt, R.C., and Nelson, R.J. 2009. Shades of gray: the world of quantitative disease resistance. *Trends Plant Sci.* 14: 21–29. doi:10.1016/j.tplants.2008.10.006.

Powder, K.E. 2020. Quantitative trait loci (QTL) mapping. *Methods Mol. Biol.* 2082: 211–229. doi:10.1007/978-1-0716-0026-9_15.

R Core Team 2021. R: A language and environment for statistical computing. R Foundation for statistical computing, Vienna, Austria, 2020; URL: https://www.r-project.org/. Accessed on: 28. 09. 2021.

Rahman, M., Mamidi, S., del Rio, L., Ross, A., Kadir, M.M., Rahaman, M.M., and Arifuzzaman, M. 2016. Association mapping in *Brassica napus* (L.) accessions identifies a major QTL for blackleg disease resistance on chromosome A01. *Mol. Breeding* 36: 90. doi:10.1007/s11032-016-0513-8.

Raman, H., Raman, R., Coombes, N., Song, J., Diffey, S., Kilian, A., Lindbeck, K., Barbulescu, D.M., Batley, J., Edwards, D., Salisbury, P.A., and Marcroft, S. 2016. Genome-wide association study identifies new loci for resistance to *Leptosphaeria maculans* in canola. *Front. Plant Sci.* 7: 1513. doi:10.3389/fpls.2016.01513.

Raman, H., Raman, R., Diffey, S., Qiu, Y., McVittie, B., Barbulescu, D.M., Salisbury, P.A., Marcroft, S., and Delourme, R. 2018. Stable quantitative resistance loci to blackleg disease in canola (*Brassica napus* L.) over continents. *Front. Plant Sci.* 9: 1622. doi:10.3389/fpls.2018.01622.

Raman, R., Diffey, S., Barbulescu, D.M., Coombes, N., Luckett, D., Salisbury, P., Cowley, R., Marcroft, S., and Raman, H. 2020. Genetic and physical mapping of loci for resistance to blackleg disease in canola (*Brassica napus* L.). *Sci Rep.* 10: 4416. doi:10.1038/s41598-020-61211-y.

Schlageter, A. 2015. Preventing wild boar *Sus scrofa* damage – considerations for wild boar management in highly fragmented agroecosystems. PhD dissertation, University of Basel, Switzerland URL:https://edoc.unibas.ch/37659/1/Thesis_A.Schlageter_Pflichtexemplar_elektronisch.pdf. Accessed on: 04. 11. 2021.

Sprague, S.J., Marcroft, S.J., Lindbeck, K.D., Ware, A.H., Khangura, R.K., and Van de Wouw, A.P. 2018. Detection, prevalence and severity of upper canopy infection on mature *Brassica napus* plants caused by *Leptosphaeria maculans* in Australia. *Crop Pasture Sci.* 69: 65. doi:10.1071/CP17140.

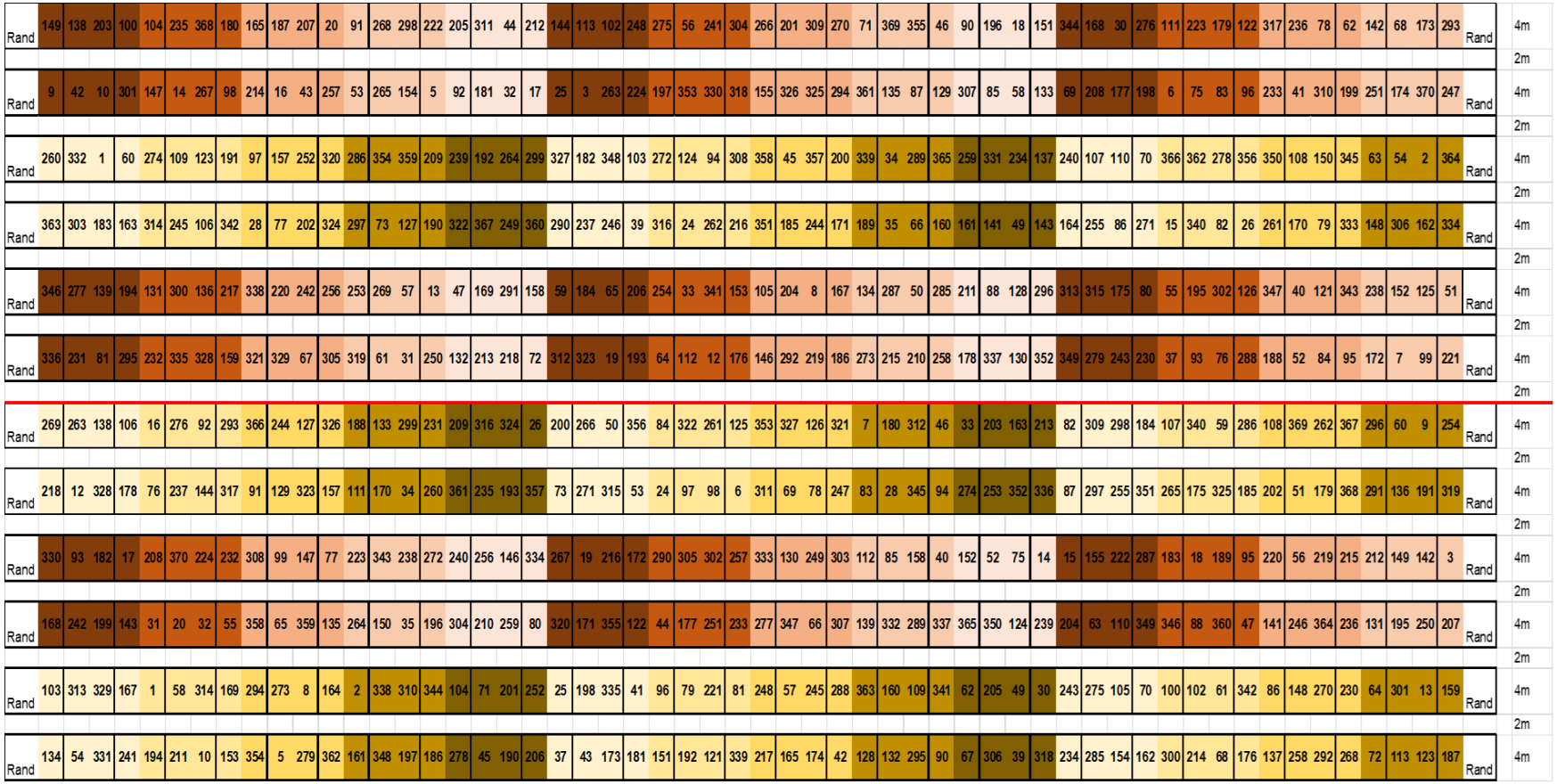
Van De Wouw, A.P., Marcroft, S.J., and Howlett, B.J. 2016. Blackleg disease of canola in Australia. *Crop Pasture Sci.* 67: 273. doi:10.1071/CP15221.

Vollrath, P., Chawla, H.S., Alnajar, D., Gabur, I., Lee, H., Weber, S., Ehrig, L., Koopmann, B., Snowdon, R.J., and Obermeier, C. 2021. Dissection of quantitative blackleg resistance reveals novel variants of resistance gene *Rlm9* in elite *Brassica napus*. *Front. Plant Sci.* 12: 749491. doi:10.3389/fpls.2021.749491.

Winter, M., and Koopmann, B. 2016. Race spectra of *Leptosphaeria maculans*, the causal agent of blackleg disease of oilseed rape, in different geographic regions in northern Germany. *Eur. J. Plant Pathol.* 145: 629–641. doi:10.1007/s10658-016-0932-8.

Zheng, X., Koopmann, B., Ulber, B., and von Tiedemann, A. 2020. A global survey on diseases and pests in oilseed rape- current challenges and innovative strategies of control. *Front. Agron.* 2: 590908. doi:10.3389/fagro.2020.590908.

# 9. Appendixes




**Appendix 1.** Field design according to the Alpha lattice design with two replications or super blocks.

**Appendix 2.** Phenotyping of the interconnected multiparent population of *B. napus* for resistance against *L.maculans* in Nienstädt, season 2017-2018.

| Genotype | VDT9 | SD. | Sig. |
|---|---|---|---|
| LO-Ga_136 | 8,16 | 1,15 | A |
| LO_AL_037 | 8,09 | 1,26 | AB |
| LO-DK_078 | 7,93 | 1,43 | AB |
| LO-Ga_142 | 7,90 | 1,47 | AB |
| LO-DH_191 | 7,89 | 1,51 | AB |
| LO-DK_095 | 7,81 | 1,58 | AB |
| LO_AL_043 | 7,77 | 1,55 | AB |
| LO-Ga_165 | 7,76 | 1,65 | AB |
| LO_AL_025 | 7,74 | 1,52 | AB |
| LO-Ga_150 | 7,71 | 1,47 | ABC |
| LO-Ga_152 | 7,60 | 2,05 | ABC |
| LO_AL_007 | 7,47 | 1,80 | ABCD |
| LO-DH_193 | 7,46 | 1,58 | ABCD |
| LO-AD_303 | 7,45 | 1,57 | ABCD |
| LO_AL_033 | 7,43 | 1,99 | ABCD |
| LO-KI_245 | 7,42 | 1,73 | ABCD |
| LO_AL_042 | 7,40 | 1,90 | ABCD |
| LO_AL_003 | 7,33 | 1,96 | ABCDE |
| LO-KI_233 | 7,30 | 1,93 | ABCDE |
| LO_AL_017 | 7,28 | 1,17 | ABCDE |
| LO-Ga_147 | 7,27 | 1,76 | ABCDEF |
| LO_AL_034 | 7,26 | 1,87 | ABCDEF |
| LO_AL_024 | 7,24 | 1,70 | ABCDEF |
| LO-DH_187 | 7,19 | 2,23 | ABCDEF |
| LO-Ga_126 | 7,17 | 1,93 | ABCDEF |
| LO-DH_192 | 7,13 | 2,09 | ABCDEF |
| LO-Ga_158 | 7,13 | 2,07 | ABCDEF |
| LO-KI_271 | 7,13 | 2,20 | ABCDEF |
| LO-DK_085 | 7,11 | 1,84 | ABCDEF |
| LO-KI_238 | 7,10 | 1,78 | ABCDEF |
| LO-DK_111 | 7,09 | 1,75 | ABCDEF |
| LO-DH_220 | 7,08 | 2,10 | ABCDEF |
| LO-AD_327 | 7,08 | 2,28 | ABCDEF |
| LO-DH_181 | 7,07 | 2,11 | ABCDEF |
| LO-KI_258 | 7,07 | 2,03 | ABCDEF |
| LO-KI_243 | 7,07 | 1,94 | ABCDEF |
| LO-AD_325 | 7,07 | 1,87 | ABCDEF |
| LO-KI_262 | 7,02 | 1,87 | ABCDEF |
| LO-KI_270 | 7,00 | 2,05 | ABCDEF |
| LO-DK_097 | 6,95 | 2,16 | ABCDEF |
| LO-DK_094 | 6,94 | 1,86 | ABCDEF |
| LO-DH_201 | 6,94 | 1,56 | ABCDEF |
| LO-DH_216 | 6,94 | 1,92 | ABCDEF |
| LO-DK_064 | 6,92 | 2,27 | ABCDEF |
| LO-DH_196 | 6,88 | 2,28 | ABCDEF |
| LO-DK_106 | 6,88 | 3,03 | ABCDEFG |
| LO-KI_236 | 6,85 | 2,35 | ABCDEFG |
| LO-DK_096 | 6,85 | 1,91 | ABCDEFG |
| LO_AL_039 | 6,85 | 1,98 | ABCDEFG |
| LO-KI_278 | 6,84 | 2,06 | ABCDEFG |
| LO-KI_242 | 6,84 | 1,70 | ABCDEFG |
| LO_AL_018 | 6,82 | 2,05 | ABCDEFG |
| LO-KI_260 | 6,81 | 2,41 | ABCDEFG |
| LO-KI_247 | 6,79 | 2,42 | ABCDEFG |
| LO-DH_197 | 6,79 | 2,05 | ABCDEFG |
| LO_AL_026 | 6,78 | 1,64 | ABCDEFG |
| LO_AL_051 | 6,77 | 2,20 | ABCDEFG |
| LO-AD_315 | 6,74 | 2,13 | ABCDEFG |
| LO-DK_091 | 6,72 | 1,92 | ABCDEFG |
| LO-KI_275 | 6,70 | 1,71 | ABCDEFG |
| LO-DH_190 | 6,67 | 2,25 | ABCDEFG |
| LO-AD_290 | 6,66 | 2,15 | ABCDEFG |
| LO-Ga_138 | 6,66 | 2,37 | ABCDEFG |
| LO_AL_060 | 6,66 | 2,14 | ABCDEFG |
| LO-DH_215 | 6,62 | 2,43 | ABCDEFG |
| LO-DK_109 | 6,60 | 2,12 | ABCDEFG |
| LO-AD_287 | 6,60 | 2,25 | ABCDEFG |
| LO-DK_098 | 6,59 | 1,99 | ABCDEFG |
| LO-DK_062 | 6,59 | 2,03 | ABCDEFG |
| LO_335 | 6,58 | 1,82 | ABCDEFG |
| LO_AL_002 | 6,57 | 1,88 | ABCDEFG |
| LO-AD_312 | 6,57 | 2,01 | ABCDEFG |
| LO-AD_314 | 6,57 | 1,67 | ABCDEFG |
| LO-KI_263 | 6,57 | 2,42 | ABCDEFG |
| LO-KI_274 | 6,56 | 2,09 | ABCDEFG |
| LO_AL_020 | 6,55 | 2,02 | ABCDEFG |
| LO-DK_099 | 6,55 | 2,08 | ABCDEFG |
| LO-DK_063 | 6,53 | 1,90 | ABCDEFG |
| LO-DK_093 | 6,50 | 2,08 | ABCDEFG |
| LO-Ga_171 | 6,48 | 1,86 | ABCDEFG |
| LO-DK_073 | 6,47 | 1,86 | ABCDEFG |
| LO-AD_331 | 6,46 | 2,30 | ABCDEFG |
| LO_AL_045 | 6,44 | 1,40 | ABCDEFG |
| LO_AL_006 | 6,44 | 1,96 | ABCDEFG |
| LO_AL_049 | 6,42 | 2,09 | ABCDEFG |
| LO-Ga_161 | 6,38 | 1,93 | ABCDEFG |
| LO-Ga_143 | 6,36 | 2,00 | ABCDEFG |
| LO_AL_041 | 6,36 | 2,46 | ABCDEFG |
| LO_AL_008 | 6,36 | 2,28 | ABCDEFG |

| | | | |
|---|---|---|---|
| LO_AL_047 | 6,35 | 1,96 | ABCDEFG |
| LO-KI_250 | 6,33 | 2,38 | ABCDEFG |
| LO-DK_066 | 6,32 | 2,13 | ABCDEFG |
| LO-DH_195 | 6,30 | 1,93 | ABCDEFG |
| LO-DH_209 | 6,29 | 2,04 | ABCDEFG |
| LO-DH_214 | 6,28 | 1,59 | ABCDEFG |
| LO-DH_210 | 6,26 | 2,70 | ABCDEFG |
| LO-AD_296 | 6,22 | 1,99 | ABCDEFG |
| LO-DK_103 | 6,21 | 2,23 | ABCDEFG |
| LO-AD_330 | 6,21 | 2,50 | ABCDEFG |
| LO-KI_254 | 6,20 | 2,65 | ABCDEFG |
| LO-AD_298 | 6,18 | 2,65 | ABCDEFG |
| LO-DH_200 | 6,17 | 2,10 | ABCDEFG |
| LO-DH_212 | 6,17 | 1,89 | ABCDEFG |
| LO_AL_056 | 6,16 | 1,95 | ABCDEFG |
| LO_AL_009 | 6,16 | 2,03 | ABCDEFG |
| LO-DH_217 | 6,14 | 2,32 | ABCDEFG |
| LO-AD_321 | 6,12 | 2,51 | ABCDEFG |
| LO_AL_005 | 6,11 | 2,32 | ABCDEFG |
| LO-DH_176 | 6,09 | 2,18 | ABCDEFG |
| LO-Ga_154 | 6,09 | 2,57 | ABCDEFG |
| LO-KI_240 | 6,09 | 2,08 | ABCDEFG |
| LO-AD_289 | 6,05 | 2,05 | ABCDEFG |
| LO-KI_252 | 6,04 | 2,81 | ABCDEFGH |
| LO-DK_076 | 6,04 | 2,64 | ABCDEFGH |
| LO_AL_010 | 6,02 | 1,82 | ABCDEFGH |
| LO-AD_291 | 6,00 | 2,70 | ABCDEFGHI |
| LO-KI_248 | 6,00 | 2,60 | BCDEFGHI |
| LO-KI_272 | 5,97 | 2,67 | BCDEFGHI |
| LO-DH_184 | 5,96 | 2,32 | BCDEFGHI |
| LO-DH_205 | 5,96 | 2,36 | BCDEFGHI |
| LO-DH_207 | 5,95 | 2,46 | BCDEFGHI |
| LO-Ga_137 | 5,95 | 2,46 | BCDEFGHI |
| LO-Ga_174 | 5,95 | 2,02 | BCDEFGHI |
| LO-DK_105 | 5,93 | 2,42 | BCDEFGHI |
| LO-DH_189 | 5,92 | 2,51 | BCDEFGHI |
| LO_AL_058 | 5,92 | 2,24 | BCDEFGHI |
| LO-DK_082 | 5,90 | 2,30 | BCDEFGHI |
| LO-Ga_131 | 5,89 | 2,14 | BCDEFGHI |
| LO_AL_057 | 5,88 | 2,22 | BCDEFGHI |
| LO-DK_077 | 5,86 | 2,09 | BCDEFGHI |
| LO-DK_092 | 5,84 | 2,27 | BCDEFGHI |
| LO-Ga_133 | 5,83 | 2,16 | BCDEFGHI |
| LO-DH_206 | 5,82 | 2,34 | BCDEFGHI |
| LO-DH_221 | 5,82 | 1,89 | BCDEFGHI |
| LO_AL_013 | 5,81 | 2,51 | BCDEFGHI |
| LO-AD_295 | 5,80 | 2,42 | BCDEFGHI |
| LO-DH_198 | 5,80 | 1,70 | BCDEFGHI |
| NK-BR_351 | 5,80 | 2,37 | BCDEFGHI |
| LO-Ga_168 | 5,79 | 2,57 | BCDEFGHI |
| LO-KI_255 | 5,79 | 2,23 | CDEFGHI |
| LO-DH_177 | 5,78 | 1,95 | CDEFGHI |
| LO-Ga_134 | 5,78 | 2,88 | CDEFGHI |
| LO-AD_326 | 5,76 | 2,21 | CDEFGHI |
| LO_AL_040 | 5,76 | 1,84 | CDEFGHI |
| LO-DH_188 | 5,76 | 2,30 | CDEFGHI |
| LO-KI_234 | 5,75 | 2,06 | CDEFGHI |
| LO-DK_110 | 5,75 | 2,43 | CDEFGHI |
| LO_AL_054 | 5,75 | 2,24 | CDEFGHI |
| LO-Ga_127 | 5,73 | 2,13 | CDEFGHI |
| LO_AL_015 | 5,72 | 2,58 | CDEFGHI |
| LO-KI_231 | 5,72 | 2,39 | CDEFGHI |
| LO-Ga_170 | 5,71 | 1,99 | CDEFGHIJ |
| LO_AL_001 | 5,70 | 2,36 | CDEFGHIJ |
| LO-AD_316 | 5,67 | 2,50 | CDEFGHIJ |
| LO-AD_329 | 5,67 | 1,76 | CDEFGHIJ |
| LO-AD_324 | 5,66 | 2,00 | CDEFGHIJ |
| LO-AD_333 | 5,64 | 2,65 | CDEFGHIJ |
| LO-Ga_121 | 5,62 | 2,18 | CDEFGHIJ |
| LO-AD_292 | 5,62 | 1,69 | CDEFGHIJ |
| LO-DK_104 | 5,59 | 2,07 | CDEFGHIJ |
| LO_AL_046 | 5,59 | 2,59 | CDEFGHIJ |
| LO-DK_069 | 5,58 | 1,81 | CDEFGHIJ |
| LO-Ga_141 | 5,58 | 2,01 | CDEFGHIJ |
| LO-DK_088 | 5,57 | 2,43 | CDEFGHIJ |
| LO-DK_070 | 5,57 | 1,72 | CDEFGHIJ |
| LO-DH_175 | 5,57 | 2,12 | CDEFGHIJ |
| LO-DH_180 | 5,56 | 2,05 | CDEFGHIJ |
| LO-DK_086 | 5,54 | 1,84 | CDEFGHIJ |
| LO-AD_328 | 5,54 | 2,62 | CDEFGHIJ |
| LO_AL_050 | 5,53 | 2,49 | CDEFGHIJ |
| LO-DH_203 | 5,52 | 1,70 | CDEFGHIJ |
| LO_AL_044 | 5,52 | 1,61 | CDEFGHIJ |
| LO-DK_065 | 5,51 | 1,89 | CDEFGHIJ |
| LO_AL_014 | 5,49 | 2,78 | CDEFGHIJ |
| LO-KI_266 | 5,48 | 2,49 | CDEFGHIJ |
| LO-AD_286 | 5,48 | 2,23 | CDEFGHIJ |
| LO-DK_075 | 5,48 | 2,54 | CDEFGHIJ |
| LO-AD_288 | 5,47 | 2,69 | CDEFGHIJ |
| LO-KI_261 | 5,45 | 2,67 | CDEFGHIJ |
| LO-AD_305 | 5,44 | 1,85 | CDEFGHIJ |
| LO-KI_267 | 5,42 | 2,58 | CDEFGHIJ |
| LO-DK_079 | 5,41 | 2,05 | CDEFGHIJ |
| LO-DH_182 | 5,41 | 2,41 | CDEFGHIJ |
| LO-DH_204 | 5,37 | 1,74 | CDEFGHIJ |
| LO-KI_277 | 5,36 | 1,99 | CDEFGHIJ |

| | | | |
|---|---|---|---|
| LO-DH_224 | 5,35 | 2,06 | CDEFGHIJ |
| LO-Ga_163 | 5,35 | 1,68 | CDEFGHIJ |
| LO-DH_178 | 5,34 | 2,42 | CDEFGHIJ |
| LO-DH_179 | 5,33 | 2,11 | CDEFGHIJ |
| LO-KI_268 | 5,33 | 2,23 | CDEFGHIJK |
| LO-DK_107 | 5,32 | 2,55 | CDEFGHIJK |
| LO-KI_276 | 5,29 | 2,11 | CDEFGHIJK |
| DH_347 | 5,28 | 2,56 | CDEFGHIJK |
| LO-KI_259 | 5,27 | 2,72 | CDEFGHIJK |
| LO_AL_053 | 5,26 | 2,59 | DEFGHIJK |
| LO-DK_071 | 5,25 | 2,40 | DEFGHIJK |
| LO-Ga_157 | 5,23 | 2,70 | DEFGHIJK |
| LO-DK_072 | 5,23 | 2,29 | DEFGHIJK |
| LO-AD_313 | 5,23 | 2,28 | DEFGHIJK |
| LO-Ga_132 | 5,22 | 2,32 | DEFGHIJK |
| LO-DH_194 | 5,21 | 2,57 | DEFGHIJKL |
| LO-Ga_151 | 5,21 | 2,60 | DEFGHIJKL |
| LO-DK_102 | 5,17 | 2,03 | DEFGHIJKL |
| LO-DH_223 | 5,16 | 2,67 | DEFGHIJKL |
| LO-AD_332 | 5,15 | 2,09 | DEFGHIJKL |
| LO-Ga_149 | 5,15 | 2,68 | DEFGHIJKL |
| LO_AL_031 | 5,14 | 2,80 | DEFGHIJKL |
| LO-KI_265 | 5,14 | 1,69 | DEFGHIJKL |
| LO-Ga_146 | 5,12 | 2,34 | DEFGHIJKL |
| LO-KI_273 | 5,10 | 2,13 | DEFGHIJKL |
| LO-DH_211 | 5,10 | 2,82 | DEFGHIJKL |
| LO-KI_256 | 5,09 | 2,03 | DEFGHIJKL |
| LO-KI_257 | 5,09 | 2,56 | DEFGHIJKL |
| LO-AD_306 | 5,08 | 2,26 | DEFGHIJKL |
| LO-Ga_153 | 5,07 | 2,44 | DEFGHIJKL |
| LO_AL_012 | 5,07 | 1,75 | DEFGHIJKL |
| LO-Ga_129 | 5,05 | 2,67 | DEFGHIJKL |
| LO-KI_239 | 5,04 | 2,80 | DEFGHIJKL |
| LO-DK_090 | 4,98 | 2,42 | DEFGHIJKL |
| LO-KI_237 | 4,93 | 3,10 | DEFGHIJKL |
| LO-DH_219 | 4,93 | 2,38 | DEFGHIJKL |
| LO-AD_297 | 4,92 | 2,27 | DEFGHIJKL |
| LO-DH_185 | 4,92 | 1,77 | DEFGHIJKL |
| LO-AD_299 | 4,92 | 2,09 | DEFGHIJKL |
| LO-KI_251 | 4,90 | 2,31 | DEFGHIJKL |
| LO-KI_279 | 4,89 | 2,57 | DEFGHIJKL |
| LO-AD_294 | 4,89 | 2,34 | DEFGHIJKL |
| LO-Ga_123 | 4,87 | 2,59 | DEFGHIJKL |
| LO-DK_061 | 4,87 | 2,40 | DEFGHIJKL |
| LO_AL_016 | 4,86 | 2,41 | EFGHIJKL |
| LO-DK_067 | 4,86 | 1,70 | EFGHIJKL |
| LO-DH_208 | 4,83 | 2,22 | EFGHIJKL |
| LO-DH_222 | 4,83 | 2,32 | EFGHIJKL |

| | | | |
|---|---|---|---|
| LO-KI_246 | 4,81 | 2,33 | EFGHIJKL |
| LO-KI_235 | 4,81 | 2,37 | EFGHIJKL |
| LO-Ga_135 | 4,80 | 1,97 | EFGHIJKL |
| LO-KI_264 | 4,80 | 2,01 | EFGHIJKL |
| LO-KI_244 | 4,78 | 2,34 | EFGHIJKL |
| LO-AD_293 | 4,78 | 2,14 | EFGHIJKL |
| LO-AD_317 | 4,77 | 2,77 | EFGHIJKL |
| LO_AL_055 | 4,77 | 2,11 | EFGHIJKL |
| LO-Ga_130 | 4,74 | 2,23 | EFGHIJKL |
| LO-Ga_169 | 4,71 | 2,68 | FGHIJKL |
| LO-AD_308 | 4,70 | 2,75 | FGHIJKL |
| KI_343 | 4,70 | 2,74 | FGHIJKL |
| LO_AL_028 | 4,68 | 2,43 | FGHIJKL |
| LO-DK_084 | 4,68 | 1,83 | FGHIJKL |
| LO-AD_322 | 4,67 | 2,54 | FGHIJKL |
| DK_345 | 4,63 | 2,57 | FGHIJKL |
| LO-KI_253 | 4,62 | 2,31 | FGHIJKL |
| LO-DH_213 | 4,61 | 2,87 | FGHIJKL |
| LO-Ga_162 | 4,60 | 2,68 | FGHIJKL |
| LO-DK_087 | 4,58 | 2,66 | FGHIJKL |
| LO-KI_230 | 4,56 | 1,93 | FGHIJKL |
| AL_337 | 4,50 | 2,74 | FGHIJKL |
| LO-AD_311 | 4,48 | 2,21 | FGHIJKL |
| LO_AL_019 | 4,48 | 1,23 | FGHIJKL |
| LO-AD_307 | 4,47 | 1,98 | FGHIJKL |
| LO-Ga_173 | 4,47 | 2,36 | FGHIJKL |
| LO_AL_052 | 4,47 | 2,56 | FGHIJKL |
| LO-DK_068 | 4,46 | 2,03 | FGHIJKL |
| LO-Ga_124 | 4,46 | 2,98 | FGHIJKL |
| LO-KI_232 | 4,45 | 2,04 | FGHIJKL |
| LO-AD_301 | 4,44 | 2,25 | FGHIJKL |
| LO-DH_186 | 4,41 | 2,18 | FGHIJKL |
| LO-Ga_139 | 4,39 | 2,55 | FGHIJKL |
| LO-AD_318 | 4,39 | 2,66 | FGHIJKL |
| LO-AD_309 | 4,37 | 1,98 | FGHIJKL |
| GA_341 | 4,35 | 2,58 | FGHIJKL |
| LO-AD_323 | 4,33 | 1,51 | FGHIJKL |
| LO-Ga_155 | 4,30 | 2,21 | FGHIJKL |
| LO-AD_320 | 4,30 | 2,17 | FGHIJKL |
| LO-Ga_167 | 4,29 | 2,37 | FGHIJKL |
| LO-DH_199 | 4,26 | 2,65 | FGHIJKL |
| LO-DK_113 | 4,25 | 2,77 | FGHIJKL |
| LO-AD_310 | 4,24 | 2,15 | FGHIJKL |
| LO-DK_100 | 4,22 | 2,12 | FGHIJKL |
| LO-AD_302 | 4,22 | 2,27 | FGHIJKL |
| LO-DK_112 | 4,21 | 2,31 | GHIJKL |
| AD_339 | 4,16 | 2,18 | GHIJKL |
| LO-DK_108 | 4,14 | 1,75 | GHIJKL |

| | | | |
|---|---|---|---|
| LO-DH_202 | 4,13 | 1,91 | GHIJKL |
| LO-AD_285 | 4,10 | 1,51 | GHIJKL |
| LO-AD_319 | 4,09 | 2,33 | GHIJKL |
| LO-DK_083 | 4,08 | 1,49 | GHIJKL |
| LO-AD_300 | 4,07 | 1,33 | GHIJKL |
| LO-AD_304 | 4,01 | 1,69 | GHIJKL |
| LO_AL_030 | 3,98 | 2,40 | GHIJKL |
| LO-Ga_144 | 3,97 | 2,05 | GHIJKL |
| LO-Ga_160 | 3,89 | 2,22 | GHIJKL |
| LO-KI_249 | 3,86 | 1,44 | GHIJKL |
| LO-AD_334 | 3,83 | 2,28 | GHIJKL |
| EXO_349 | 3,81 | 2,61 | GHIJKL |
| LO-Ga_148 | 3,76 | 2,45 | GHIJKL |
| LO-Ga_128 | 3,74 | 1,88 | GHIJKL |
| LO-DK_080 | 3,61 | 2,09 | GHIJKL |
| LO-Ga_172 | 3,57 | 1,89 | GHIJKL |
| LO_AL_059 | 3,52 | 2,16 | GHIJKL |
| LO-DK_081 | 3,51 | 2,08 | GHIJKL |
| LO-KI_269 | 3,47 | 2,68 | HIJKL |
| LO-Ga_122 | 3,43 | 2,60 | HIJKL |
| LO_AL_035 | 3,38 | 1,10 | HIJKL |
| LO-DH_218 | 3,18 | 1,53 | IJKL |
| LO-KI_241 | 3,16 | 2,02 | IJKL |
| LO-Ga_159 | 3,15 | 2,35 | JKL |
| LO-Ga_125 | 2,94 | 1,35 | KL |
| LO-Ga_164 | 2,75 | 2,27 | L |
| LO-DH_183 | 2,51 | 1,61 | L |

frontiers in Plant Science

ORIGINAL RESEARCH
published: 18 November 2021
doi: 10.3389/fpls.2021.749491

# Dissection of Quantitative Blackleg Resistance Reveals Novel Variants of Resistance Gene *Rlm9* in Elite *Brassica napus*

*Paul Vollrath[1], Harmeet S. Chawla[2], Dima Alnajar[3], Iulian Gabur[1,4], HueyTyng Lee[1], Sven Weber[1], Lennard Ehrig[1], Birger Koopmann[3], Rod J. Snowdon[1] and Christian Obermeier[1]**

[1] Department of Plant Breeding, IFZ Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University Giessen, Giessen, Germany, [2] Department of Plant Sciences, Crop Development Centre, University of Saskatchewan, Saskatoon, SK, Canada, [3] Plant Pathology and Crop Protection Division, Department of Crop Sciences, Georg August University of Göttingen, Göttingen, Germany, [4] Department of Plant Sciences, Faculty of Agriculture, Iasi University of Life Sciences, Iaşi, Romania

Blackleg is one of the major fungal diseases in oilseed rape/canola worldwide. Most commercial cultivars carry *R* gene-mediated qualitative resistances that confer a high level of race-specific protection against *Leptosphaeria maculans*, the causal fungus of blackleg disease. However, monogenic resistances of this kind can potentially be rapidly overcome by mutations in the pathogen's avirulence genes. To counteract pathogen adaptation in this evolutionary arms race, there is a tremendous demand for quantitative background resistance to enhance durability and efficacy of blackleg resistance in oilseed rape. In this study, we characterized genomic regions contributing to quantitative *L. maculans* resistance by genome-wide association studies in a multiparental mapping population derived from six parental elite varieties exhibiting quantitative resistance, which were all crossed to one common susceptible parental elite variety. Resistance was screened using a fungal isolate with no corresponding avirulence (*AvrLm*) to major *R* genes present in the parents of the mapping population. Genome-wide association studies revealed eight significantly associated quantitative trait loci (QTL) on chromosomes A07 and A09, with small effects explaining 3–6% of the phenotypic variance. Unexpectedly, the qualitative blackleg resistance gene *Rlm9* was found to be located within a resistance-associated haploblock on chromosome A07. Furthermore, long-range sequence data spanning this haploblock revealed high levels of single-nucleotide and structural variants within the *Rlm9* coding sequence among the parents of the mapping population. The results suggest that novel variants of *Rlm9* could play a previously unknown role in expression of quantitative disease resistance in oilseed rape.

**Keywords: ONT, structural variation, blackleg, *Brassica napus*, long-read sequencing, Rlm9**

OPEN ACCESS

***Edited by:***
*Jacqueline Batley, University of Western Australia, Australia*

***Reviewed by:***
*Angela Van De Wouw, The University of Melbourne, Australia*
*Francis Chuks Ogbonnaya, Grains Research and Development Corporation, Australia*

****Correspondence:***
*Christian Obermeier*
*christian.obermeier@agrar.uni-giessen.de*

***Specialty section:***
*This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science*

***Received:*** *29 July 2021*
***Accepted:*** *29 October 2021*
***Published:*** *18 November 2021*

***Citation:***
*Vollrath P, Chawla HS, Alnajar D, Gabur I, Lee H, Weber S, Ehrig L, Koopmann B, Snowdon RJ and Obermeier C (2021) Dissection of Quantitative Blackleg Resistance Reveals Novel Variants of Resistance Gene Rlm9 in Elite Brassica napus. Front. Plant Sci. 12:749491. doi: 10.3389/fpls.2021.749491*

## 1. INTRODUCTION

Oilseed rape/canola (*Brassica napus* L.) is one of the most important vegetable oil crops. As a recent allotetraploid crop, originating from an interspecific hybridization event between its two diploid ancestors *Brassica rapa* (2n = 2x = 20, AA) and *Brassica oleracea* (2n = 2x = 18, CC) (Nagaharu, 1935), *B. napus* (2n = 4x = 38, AACC) carries a highly complex and dynamic genome which is

affected by many small-scale and large-scale structural variations (Parkin et al., 1995; Chalhoub et al., 2014; Stein et al., 2017; Hurgobin et al., 2018; Chawla et al., 2020). Many studies revealed high frequencies of homoeologous exchanges between the highly similar A and C subgenomes (Szadkowski et al., 2010; Chalhoub et al., 2014; Samans et al., 2017).

The hemibiotrophic fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & de Not. [anamorph: *Phoma lingam* (Tode ex. Fr.) Desm.] causes stem canker in *B. napus*. This disease, also known as blackleg, is a major problem in almost all oilseed rape and canola-growing regions around the globe. Substantial yield losses have been reported in Australia, North America and several European countries (Fitt et al., 2006). The primary infection of winter oilseed rape takes place in autumn via airborne ascospores. Additionally, secondary infections are likely through pycnidiospores formed within the asexual pycnidia. The spores penetrate the host tissue via stomata or wounds and colonize intercellular spaces of the mesophyll. From there, the fungus starts its symptomless, biotrophic growth systematically through the petiole into the stem. Here the pathogen kills the cells, leading to girdling and rotting of the stem base. As a result, the plant tends to ripen prematurely and severe infections can lead to serious lodging and death (West et al., 2001). Resistance breeding is the most sustainable and effective method to counteract *L. maculans*. Resistance of *B. napus* against *L. maculans* is often divided into two classes of resistance mechanisms. A distinction is made between race-specific, qualitative resistance determined by major genes, and non-race-specific quantitative resistance provided by numerous minor effect genes. Qualitative resistance against *L. maculans*, considered as complete resistance, has been investigated in considerable detail and used extensively in commercial breeding programs of *B. napus*, due to the high efficacy and convenient assessment at the cotyledon stage (Rimmer and van den Berg, 1992; Balesdent et al., 2005; Delourme et al., 2006; Elliott et al., 2016). However, it has been observed that rapid adaptation of the pathogen populations can overcome *R* gene-mediated resistance in the field within a few seasons (Rouxel et al., 2003; Sprague et al., 2006; Brun et al., 2010; Zhang et al., 2016; van de Wouw et al., 2017). Thus, commercial breeders place special focus on quantitative disease resistance. Quantitative resistance is influenced by multiple genes, and the incomplete nature of the resistance decreases the selection pressure on the pathogen population and consequently increases the durability of the resistance (St Clair, 2010; Delourme et al., 2014). In contrast to qualitative resistance, the assessment of quantitative resistance is more challenging as it is mainly expressed at adult plant stages and highly influenced by environmental conditions (Fitt et al., 2006; Huang et al., 2009). A main target of breeding is therefore the combination of highly effective *R* gene-mediated resistance with a broad and durable quantitative resistance (Brun et al., 2010; Pilet-Nayel et al., 2017). However, this clear distinction between the two types of resistance has recently been questioned in several studies (Thomma et al., 2011; Delplace et al., 2020). Also, in some cases the expression of partial resistance in adult plants imparted by major resistance genes has been demonstrated (Chantret et al., 1999; Raman et al., 2018). The complexity of the *B. napus–L. maculans* pathosystem and possible corresponding genes often makes the assessment of quantitative resistance difficult. Therefore, the objective of the present study was to identify genomic regions involved in quantitative blackleg resistance by excluding the possibility of effective *R* gene-*Avr* gene interactions. A multiparental mapping population was tested under controlled greenhouse conditions with a selected highly virulent fungal isolate. In addition, whole-genome long-read re-sequencing using Oxford Nanopore Technology (ONT) was conducted to reveal the implications of single nucleotide variants (SNV) and structural genome variations (SV) on an agronomical highly important trait within the narrow genepool of European elite winter oilseed rape. Recently, Chawla et al. (2020) demonstrated a previously unknown extent of genome-wide, small to medium-sized SV events within *B. napus* genes using ONT long-read sequencing technology. In contrast, SNV have to date been largely ignored in quantitative trait analysis of important crop traits, due to the difficulty of assaying SV on a genome-wide scale in complex crop genomes. In the past few years, however, rapidly decreasing costs and increasing accuracy of long-read sequencing from the ONT or Pacific Biosciences platforms has opened the way to include genome-wide SV data from long-read sequences in QTL analysis and interpretation. Here we successfully called SNV in ONT data from the seven elite winter oilseed rape parents of the multiparental mapping population, enabling us to associate genome-wide SV with single nucleotide polymorphism (SNP) haplotypes carrying blackleg resistance QTL.

## 2. MATERIALS AND METHODS

### Plant Material

A *B. napus* multiparental population comprising 354 double haploid (DH) lines derived from seven European elite winter oilseed rape varieties was tested for quantitative blackleg resistance in multiple greenhouse screenings. The mapping population consisted of six subfamilies derived from crosses of the elite parent "Lorenz" to six elite founder lines ("Adriana," "Alpaga," "DK Cabernet," "Galileo," "King 10," and the DH line "JN"). Each of the subfamilies comprised 60 DH lines except for the cross Lorenz × Galileo, which comprised 54 DH lines. The common parent Lorenz was previously classified as highly susceptible to blackleg disease, whereas the other six founder lines were all known to carry quantitative blackleg resistance (unpublished data, breeding companies). The German breeding companies NPZ Innovation GmbH (Holtsee, Germany), Syngenta Seeds GmbH (Bad Salzuflen, Germany), and KWS SAAT SE & Co. KGaA (Einbeck, Germany) produced and provided the DH families. In parallel, another panel of 256 diverse winter oilseed rape inbred lines was tested for blackleg resistance in a 2-year field trial with one plot per field. These accessions were part of the ERANET-ASSYST *B. napus* diversity set, previously described by Bus et al. (2011).

## Resistance Screenings and Data Analysis

The multiparental mapping population was screened for adult-plant blackleg resistance under controlled conditions in the greenhouse of Georg August University of Göttingen in 11 independent screening experiments, each individual screening included all 354 genotypes with two plant individuals (replicates) per genotype in a completely randomized design. In total 22 plant individuals were tested per genotype across 11 screening experiments (22 replicates per genotype). Manual infection was carried out at developmental stage BBCH 13–14 (3–4 true leaves, leaf pairs, or whorls unfolded) (Lancashire et al., 1991). *L. maculans* was propagated on oatmeal agar medium 2 weeks before infection. A mycelial agar plug was then placed at the stem base, slightly above the axil of the first true leaf, after wounding of the infection site using a needle. Subsequently, plants were grown under foil tunnels for 72 h to ensure appropriate humidity and temperature for a successful infection. At 49 days post infection (dpi), a cross section was cut at the stem base to estimate the length (L), girdling (G), and penetration depth (P) of the blackleg lesions. L was measured in mm whereas G and P were visually scored as percentage of the total circumference and diameter, respectively. Next, these scores were converted into individual 0–9 scales for each score. Using L, G, and P scoring values at 49 dpi, the Volume of Diseased Tissue (VDT) value was calculated using a formula modified from Kutcher et al. (1993):

$$VDT = \left(1 - (\frac{1-P}{9})^2\right) * \frac{G}{9} * L$$

Also, single screening means of the two replicates per screening, adjusted means across all the eleven screenings, were calculated using the R packages lmerTest version 3.1-2 (Kuznetsova et al., 2017) and lsmeans version 2.30-0 (Lenth, 2016). This approach allowed the assessment of QTL stability across different screenings.

The diversity set was grown in 2016/2017 and 2017/2018 in field trials in Rauischholzhausen, Germany. It was grown under normal farming practices with no use of fungicides. The fields were chosen based on close crop rotation and known high natural blackleg infection pressure. Single plots per genotype were sown and analyzed in a randomized complete block design, with plot sizes of 12.5 $m^2$ (10 m × 1.25 m). At developmental stage BBCH 83–85 (30–50% of pods ripe, seeds black, and hard), 20 plants from the middle row of each plot were uprooted and cut at the stem base. Visual scores from 1 to 6 for blackleg infestation at the resulting cross section were used to calculate the G2 index for blackleg adult plant stem infection (Pilet et al., 1998; Aubertot et al., 2004) using the formula:

$$G2\ index = [(N1x0) + (N2x1) + (N3x3) + (N4x5) + (N5x7) + (N6x9)]\ /Nt,$$

where N1, N2, N3, N4, N5, and N6 are the number of stems with scores 1, 2, 3, 4, 5, and 6, respectively, and Nt is the total number of stems assessed.

## Characterization of Fungal Isolates

Leaf samples with characteristic lesions of *L. maculans* were collected in the field trials and dried. Leave segments were incubated in humid chambers to allow spore release from pycnidia. Single pycnidium isolates were prepared by plating spores on SNA medium amended with 200 ppm streptomycin for 6 days. Petri dishes were incubated under UV light at 20°C. Subsequently, a mycelial plug was transferred to V8 medium supplemented with 200 ppm streptomycin and incubated for 2 weeks under the same conditions. Spore suspensions were prepared by adjustment to $1 \times 10^7$ spores/ml using a hemocytometer. To characterize *L. maculans* isolates, cotyledon tests were applied using a differential set of *B. napus* harboring major resistance genes (**Supplementary Table 3**; Balesdent et al., 2002, 2006; Marcroft et al., 2012). Shortly, 10 µl spore suspension was applied on each lobe of cotyledon after injuring it with a needle. Eight replications were used. Symptoms were evaluated 14 days post inoculation according to the IMASCORE rating scale, where class 1 shows typical hypersensitive reactions and class 6 reflects tissue collapse with sporulation. Classes 1–3 were considered as resistance reactions, while classes 4–6 were noted as susceptible ones (Balesdent et al., 2001).

## Single Nucleotide Polymorphism Genotyping and Analysis of Linkage Disequilibrium

All the investigated *B. napus* accessions were genotyped using the *Brassica* 60k Illumina Infinium™ SNP array. The *B. napus* reference genome assembly Darmor-*bzh* version 10 (Rousseau-Gueutin et al., 2020) was used to anchor 34,079 markers uniquely to a single position of the genome (**Supplementary Table 1**). Markers mapping unspecifically to multiple positions were excluded from further analyses. Finally, single hits were filtered for a cut-off *e*-value of $1e^{-15}$. Heterozygote SNP calls were considered as missing data since we should not expect heterozygote calls for DH or inbred lines, hence it can be assumed that these calls are mostly due to technical artifacts. Genome-wide linkage disequilibrium (LD) was calculated using the R package SelectionTools version 19.4[1]. Prior to LD analysis, markers were filtered for minor allele frequency (MAF) ≥0.05 and a maximum of 10% missing data per marker and DH line. A tolerance threshold of $r^2 > 0.4$ was set to assign markers in strong LD to respective LD blocks.

## Genome-Wide Association Studies

Genome-wide association studies were conducted using the R package GenABEL version 1.8-0 (Aulchenko et al., 2007). Markers for the multiparental population were filtered as described above for LD analysis. This approach led to a set of 17,869 polymorphic and unique markers, including 16,400 SNP markers and 1,469 Single Nucleotide absence Polymorphism (SNaP) markers called as described by Gabur et al. (2018). The linear mixed model was adjusted for population structure by consideration of identity-by-state estimates and the first

[1]http://population-genetics.uni-giessen.de/~software/

two principal components (PC) as covariates. To reduce false positive rates, LD blocks were determined as suggestive QTL when a minimum of two markers per block showed trait associations in at least two individual greenhouse screening rounds. A LOD score of $-\log10$ ($p$-value) $\geq 3.0$ was applied as threshold for suggestive marker-trait associations. Finally, QTL were determined after correction for false discovery rate (FDR $\leq 0.1$) when performing GWAS with the adjusted means across all greenhouse screening rounds.

In order to validate QTL discovered in the multiparental population using the greenhouse data, we also performed an independent GWAS in the diversity panel using phenotypic data from the field trials. Similar filtering steps led to 23,603 polymorphic SNP markers. Here, LD-based QTL were defined by considering kinship and PC and applying a LOD score of $-\log10$ ($p$-value) $\geq 3.0$ as an arbitrary threshold for putative marker-trait associations.

## Functional Annotation of Darmor-*bzh* Genes

Functional annotation data for Darmor-*bzh* v4.1 produced by Gabur et al. (2020) were used together with genome-wide functional annotation of Darmor-*bzh* v10 performed using the "Automatic assignment of Human Readable Descriptions" (AHRD)[2] package (**Supplementary Table 2**). AHRD obtains the functional annotations for gene models by blasting them to various publicly available protein databases such as Swiss-Prot, TAIR or trEMBL. Two hundred best scoring blast results (based on *e*-value) were chosen from each of the above-mentioned databases. Description for all the resulting blast hits was then assigned a score using a multi-step approach. In the first step every description line was subjected to a couple of regular expression filters, removing descriptions such as "Whole genome shotgun sequence" and other vague terms like "OS = Arabidopsis thaliana." In the subsequent step the description lines were broken down into single tokens. These tokens were then pushed through a blacklist filter, thereby discarding all the tokens present in the blacklist. Every token was then assigned an overlap score based on the bit score, the database score, and the overlap score of the blast match. In the last step the token score was divided by a correction factor to remove any bias toward longer or shorter description lines. For the exact database and software versions please refer to the "Darmor10_input_go_prediction.yaml" file in the **Supplementary Material**.

## Whole Genome Long-Read Resequencing and Variant Calling

Long-read sequencing was performed for all the seven parental lines of the mapping population using Oxford Nanopore Technologies (ONT). DNA extraction was conducted as described in Chawla et al. (2020). In addition, DNA size selection was performed using the Circulomics Short Read Eliminator Kit (Circulomics Inc.). The recommended kit LSK-109 from ONT was used for DNA library preparation. Long-read sequencing was performed using the MinION device from ONT. Subsequently, basecalling was executed with Guppy version 4.0.14 and reads were aligned to the *B. napus* reference assembly Darmor-*bzh* v10 using the long-read mapper NGMLR version 0.2.7 (Sedlazeck et al., 2018). BAM files were created using samtools version 1.9 (Li et al., 2009). For genome-wide SV detection the variant caller Sniffles version 1.0.12 was used with default settings (Sedlazeck et al., 2018). Deletions and insertions with a minimum size of 30 bp were classified as SV. In addition, SNV calling was conducted using the deep neural network based variant caller Clair version 2 (Luo et al., 2020). The Clair module callVarBam, with a model for ONT data, was used to call SNV from BAM files. To reduce false positive calls, SNV calls were filtered for a minimum quality using a shell script kindly provided by Fritz Sedlazeck and Medhat Mahmoud, Baylor College of Medicine, Human Genome Sequencing Center, Houston, TX, United States. The script calculates the most appropriate cut-off value for SNV quality filtering according to the recommendations of the authors of Clair. Luo et al. (2020) observed that quality scores of variants derived from ONT data are usually bimodally distributed, so that high-quality base calls can be extracted by setting a quality cut-off at a value corresponding to the bottom of the valley between the two peaks plus 50. In addition to quality filtering, a stringent filtering for only homozygous calls and a minimum allele frequency (AF) of 0.5 for the variant were applied. Then, the files of the single genotypes were merged using bcftools version 1.10.2 and were subsequently used as an input to invoke the force-calling parameter of Clair. Next, all samples were run again to force-call the SNV provided within the merged file. This again was followed by filtering for quality, homozygous calls and a minimum AF of 0.1. A lower threshold for AF was chosen after force-calling, since previous SNV calling already indicates the presence of these variants. Subsequently, CLC Genomics Workbench (v9.0, QIAGEN Digital Insights, Aarhus, Denmark) was used to align the sequences of the parental lines and to predict the impacts of amino acid changes caused by SNV.

## Single Nucleotide Variants Validation

Due to the high number of SNV, the sequence of the gene A07p27010.1_BnaDAR was selected to validate the SNV calling method. For this purpose, sets of primers were designed to amplify the selected region and Sanger Sequencing was performed in the mapping parents. Primers were designed using the online tool Primer3Plus (Untergasser et al., 2007).

# 3. RESULTS

## Identification of a *L. maculans* Isolate Without *R* Gene Interaction for Use in Quantitative Resistance Screening

From the 644 *L. maculans* isolates collected in Northern Germany and characterized by Winter and Koopmann (2016), one isolate was selected (isolate 1.4.1.15). This isolate has been shown in cotyledon test with a *B. napus* differential set to harbor virulence alleles against *R* genes *Rlm1*, *Rlm2*, *Rlm4*, *Rlm7*, *Rlm9, LepR2,* and *LepR3*. The isolate was tested on the cotyledons of the

[2]https://github.com/groupschoof/AHRD

seven elite oilseed rape parents of the multiparental mapping population. It showed a susceptible interaction in all cotyledon tests indicating that none of the parents exhibit any qualitative major resistance against this fungal isolate based on plant *R* gene and fungal avirulence gene interaction (**Supplementary Table 3**). Thus, this virulent isolate 1.4.1.15 recovered from a field in Peine (Germany) in 2013 was used subsequently for the greenhouse screenings for quantitative resistance, in order to avoid interaction of major monogenic resistance genes with fungal avirulence genes that could putatively mask the effects of minor quantitative resistance loci in the mapping population.

## European Elite *B. napus* Accessions Show Genetic Variation for Quantitative Blackleg Resistance

Mixed linear models (MLM) demonstrated significant genotypic variation within the multiparental population ($p < 0.001$). VDT values from greenhouse screenings showed a normal distribution within the six subfamilies as well as in the entire mapping population, confirming the quantitative inheritance of blackleg resistance in this population (**Supplementary Figure 1**). LD analysis resulted in an average number of 33 LD blocks per chromosome. Single greenhouse screening rounds using mean VDT values from 2 replicates per genotype revealed together up to 326 marker-trait associations in GWAS. GWAS using mean VDT values composed of 22 replicates for each genotype merged from a total number of 11 screening rounds identified 84 significant marker-trait associations (**Supplementary Table 4**). After FDR correction a total of eight QTL regions were identified (**Table 1**), seven on chromosome A09 and one on chromosome A07. No QTL were detected on C-subgenome chromosomes. As expected for quantitative resistance, phenotypic variation explained by individual SNP markers was low and was ranging from 3.2 to 5.6% (**Table 1**). QTL stacking in the multiparental population revealed that the allele combination present in the common parent Lorenz led to the highest susceptibility compared to alleles from the other parental lines. This confirmed the initial assumption and the choice of Lorenz as the susceptible common parent. Almost all resistance alleles were derived from the six founder lines, whereas only one resistance allele was derived from the common parent Lorenz. The ideal allele combination of the eight QTL resulted in an estimated effect on resistance of 35.5% (−2.06 on the VDT scale). This beneficial combination was already present in the two founder lines DK Cabernet and JN (**Supplementary Table 5**).

## Candidate Gene Analysis for Genes Involved in Quantitative Resistance

Based on two approaches together we identified 128 genes across the eight QTL regions that are associated to defense response and/or resistance in at least one reference genome (**Supplementary Table 6**). The QTL on chromosome A07 (haploblock A07.b304) was detected in the highest number of individual screening rounds (**Table 1**). The haploblock had a size of 888 kb in the investigated multiparental mapping population and contained 53 SNP markers and 193 genes in

**TABLE 1 |** Blackleg resistance QTL (VDT trait) identified in a *B. napus* multiparental elite mapping population in 11 independent greenhouse screening rounds ($n = 354$) and overlapping resistance QTL (G2 or stem lesion) in the ERANET-ASSYST diversity set in two field trials ($n = 256$).

| | | Multiparental elite mapping population | | | | | ERANET-ASSYST diversity set | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **QTL ID** | **Chromo-some** | **Start-end position of LD block/QTL** | **Size of QTL** | **LOD#** | **$R^2$#** | **Detection in screening rounds*** | **Start-end position of LD block/QTL** | **Size of QTL** | **LOD#** | **$R^2$#** | **Detection in field trial** |
| A07.b304 | A07 | 20,107,508–20,995,393 | 888 kb | 3.35 | 3.40% | T, 3, 6, 7, 9, 10 | 20,079,904–20,378,231 | 298 kb | 3.57 | 7.79% | 2017/18 |
| A09.b366 | A09 | 13,704,742–14,702,671 | 998 kb | 5.17 | 5.48% | T, 1, 3, 7 | 12,981,315–14,141,992 | 1,160 kb | 3.46 | 6.82% | 2016/17 |
| A09.b369 | A09 | 15,852,358–16,237,668 | 385 kb | 5.22 | 5.53% | T, 1, 3, 7 | ns | ns | ns | ns | – |
| A09.b370 | A09 | 16,917,123–16,939,597 | 22 kb | 3.17 | 3.19% | T, 1, 3 | ns | ns | ns | ns | – |
| A09.b374 | A09 | 18,825,078–19,494,241 | 669 kb | 5.07 | 5.36% | T, 2, 3, 11 | ns | ns | ns | ns | – |
| A09.b380 | A09 | 33,761,934–33,966,136 | 204 kb | 5.02 | 5.32% | T, 1, 2, 3, 11 | ns | ns | ns | ns | – |
| A09.b381 | A09 | 34,149,762–38,832,384 | 4,683 kb | 3.50 | 3.58% | T, 1, 3 | ns | ns | ns | ns | – |
| A09.b382 | A09 | 38,844,647–41,182,649 | 2,338 kb | 3.88 | 4.09% | T, 1, 3 | 41,110,836–42,454,513 | 1,343 kb | 3.07 | 5.53% | 2016/17 |

*#LOD and $R^2$ values for peak markers; *T = data from 11 screening rounds combined (adjusted mean values), numbers indicate different screening rounds with 2 plant individuals per genotype; ns, not significant.*

Darmor-*bzh* v10 (**Supplementary Table 7**). However, some genes in the interval revealed no annotation in Darmor-*bzh* v4.1 because no homologous genes exist between Darmor-*bzh* v4.1 and v10 or because Blast2GO revealed no annotation for the Darmor-*bzh* v4.1 genes (60 of 193). Thus, the protein sequences of the 193 *B. napus* Darmor-*bzh* v10 genes from the QTL interval were additionally aligned to the Arabidopsis reference genome Araport11 (Cheng et al., 2017) and literature links were evaluated exhibiting some additional, more detailed functional annotations. Out of 193 genes, 17 were associated to defense response and/or resistance (**Supplementary Table 7**). In particular, we found substantiated evidence in the literature that three of these 17 genes have a function related to fungal plant resistance in *A. thaliana* and *B. napus* in interaction with common *B. napus* fungal pathogens (A07p26890.1_BnaDAR, A07p28430.1_BnaDAR, A07p27010.1_BnaDAR). Most interestingly, the gene A07p27010.1_BnaDAR has been shown to be the major resistance gene *Rlm9*, which imparts qualitative resistance against *L. maculans* in *B. napus* (Larkan et al., 2020). To evaluate for the presence of resistance gene analogs (RGAs) in the QTL region of A07.b304 and their polymorphisms between the parents of the multiparental mapping population, we also located the QTL interval for Darmor-*bzh* versions 4.1 (Chalhoub et al., 2014) and 8.1 (Bayer et al., 2017) based on the flanking SNP markers (**Supplementary Table 8**). However, two out of five putative RGAs identified in this interval showed no polymorphism and none of them had been annotated to be involved in plant resistance.

## Identification of Major Resistance Gene *Rlm9* as Candidate Gene for Quantitative Blackleg Resistance

The detected quantitative resistance region has been mapped before for the qualitative resistance gene *Rlm9* in young seedlings (Larkan et al., 2016b; Raman H. et al., 2020). However, testing of virulence complexity proved that the isolate used for greenhouse screening for quantitative resistance in our experiments shows no gene-for-gene interaction with *Rlm1*, *Rlm2*, *Rlm4*, *Rlm7*, *Rlm9, LepR2,* and *LepR3* (see above). On the other hand, variant calling using ONT long-read sequencing data revealed that the sequence diversity in the gene A07p27010.1_BnaDAR (*Rlm9*) was considerably higher than in all other genes within the QTL interval and all putative resistance genes in any of the other identified QTL (**Supplementary Table 7**). Within the QTL interval, the peak SNP marker lies just 35 kb away from the *Rlm9* gene. Within the sequence of *Rlm9*, we found 142 polymorphic SNV calls and a 6 kb insertion in four of the seven parental lines (Adriana, Alpaga, Galileo, and King 10). Parental lines Lorenz (susceptible), DK Cabernet and JN displayed an identical haplotype, comprising 24 SNPs in LD to the peak SNP marker, whereas the resistant genotypes harboring the insertion within the sequence of *Rlm9* (Adriana, Alpaga, Galileo, King 10) showed a deviating haplotype (**Figure 1**). PCR and Sanger sequencing proved both, the authenticity of the 6 kb insertion and the correctness of the 142 intragenic SNV calls in A07p27010.1_BnaDAR. Each of the 142 SNV calls from ONT data was confirmed to be correct, whereby the Sanger sequencing also revealed a further 60 SNV within this particular gene. These 60 false negatives can be explained by the strict filtering process applied to eliminate false positive calls in the SNV calling approach. Nevertheless, the results indicate that SNV calling from "noisy" long reads produced with ONT provides reliable variant calls for genetic analysis.

*Rlm9* codes for a wall-associated kinase like (WAKL) protein. A search for motifs using the Conserved Domain Search tool implemented in NCBI along with the Pfam database revealed three conserved domains, two within exon 1 and one within exon 3. These comprise an extracellular galacturonan-binding domain (GUB_WAK), a C-terminal wall-associated kinase (WAK) and an intracellular Serine/Threonine protein kinase domain (Ser/Thr_kinase). In addition, an EGF-like domain is located in exon 2 (Larkan et al., 2020). Based on the Sanger sequencing we found that three of the seven parental genotypes (Lorenz, DK Cabernet, JN) carry an identical *Rlm9* allele to Darmor-*bzh*, whereas the other four harbor a 6 kb insertion within the second exon along with 202 SNV throughout the entire gene (Adriana, Alpaga, Galileo, King 10). These SNV caused in total 92 non-synonymous amino acid changes. We observed 19 amino acid changes within the GUB_WAK domain (84.2% identity to Darmor-*bzh*), 15 amino acid changes within the WAK domain (86.7% identity to Darmor-*bzh*) and 23 amino acid changes as well as a stop codon within the Ser/Thr_kinase domain (91.3% identity to Darmor-*bzh*). The EGF-like domain in exon 2 was disrupted by the 6 kb insertion in genotypes Adriana, Alpaga, Galileo, and King 10 (**Figure 2**).

## Candidate Genes in Co-localizing Quantitative Trait Loci From Greenhouse and Field Trials

The QTL regions and candidate genes where initially identified in the elite population under controlled conditions excluding *R* gene interaction. To putatively support these candidate genes, we also identified QTL for adult plant resistance under field conditions in a diversity set. The field trials using the ERANET-ASSYST diversity set relied on natural infection. Characterization of field isolates sampled at the field site in Rauischholzhausen (Germany) revealed fungal isolates harboring the avirulence alleles *AvrLm3* or *AvrLm7* in combination with virulence alleles *avrLm1, avrLm2, avrLm4,* and *avrLm9*. This situation represents the typical German field situation as reported previously (Winter and Koopmann, 2016). This implies that monogenic *Rlm3* and *Rlm7*, also located on chromosome A07 (Larkan et al., 2016b; Raman R. et al., 2020) and potentially harbored by some of the genotypes of the diversity set, could mask quantitative resistance associated with the investigated genomic region on chromosome A07. However, monogenic *Rlm9* effects should not mask quantitative resistance effects associated with *Rlm9* in this field situation. Three of the eight QTL found in the analysis with the multiparental mapping population were also found in field trials for blackleg stem lesions in the ERANET-ASSYST diversity set (QTL A07.b304, A09.b366 and A09.b382, **Table 1** and **Supplementary Table 9**). The LD blocks for these three QTL

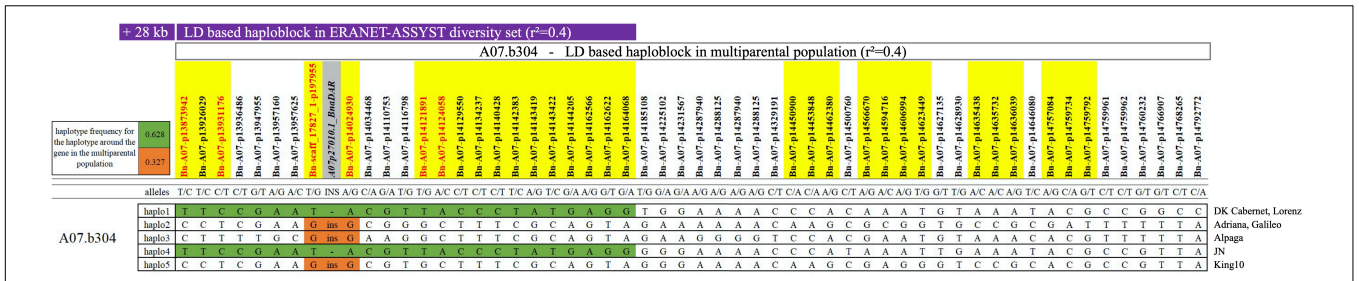



**FIGURE 1 |** LD based haploblock A07.b304 ($r^2$ = 0.4) containing 54 single nucleotide polymorphism (SNP) markers and *Rlm9* (A07p27010.1_BnaDAR). Green color indicates the two haplotypes with 24 identical SNP markers between the three parental lines Lorenz, DK Cabernet and JN. Haplotypes 2, 3, and 5 are harboring the 6 kb insertion in the sequence of *Rlm9* (orange). Purple bar highlights the overlap with the LD based QTL identified in the diversity set in field trials. Gray highlight indicates Rlm9 gene, yellow highlight indicates SNP markers identified in one individual greenhouse screening round, red color indicates SNP markers identified in at least two individual greenhouse screening rounds.

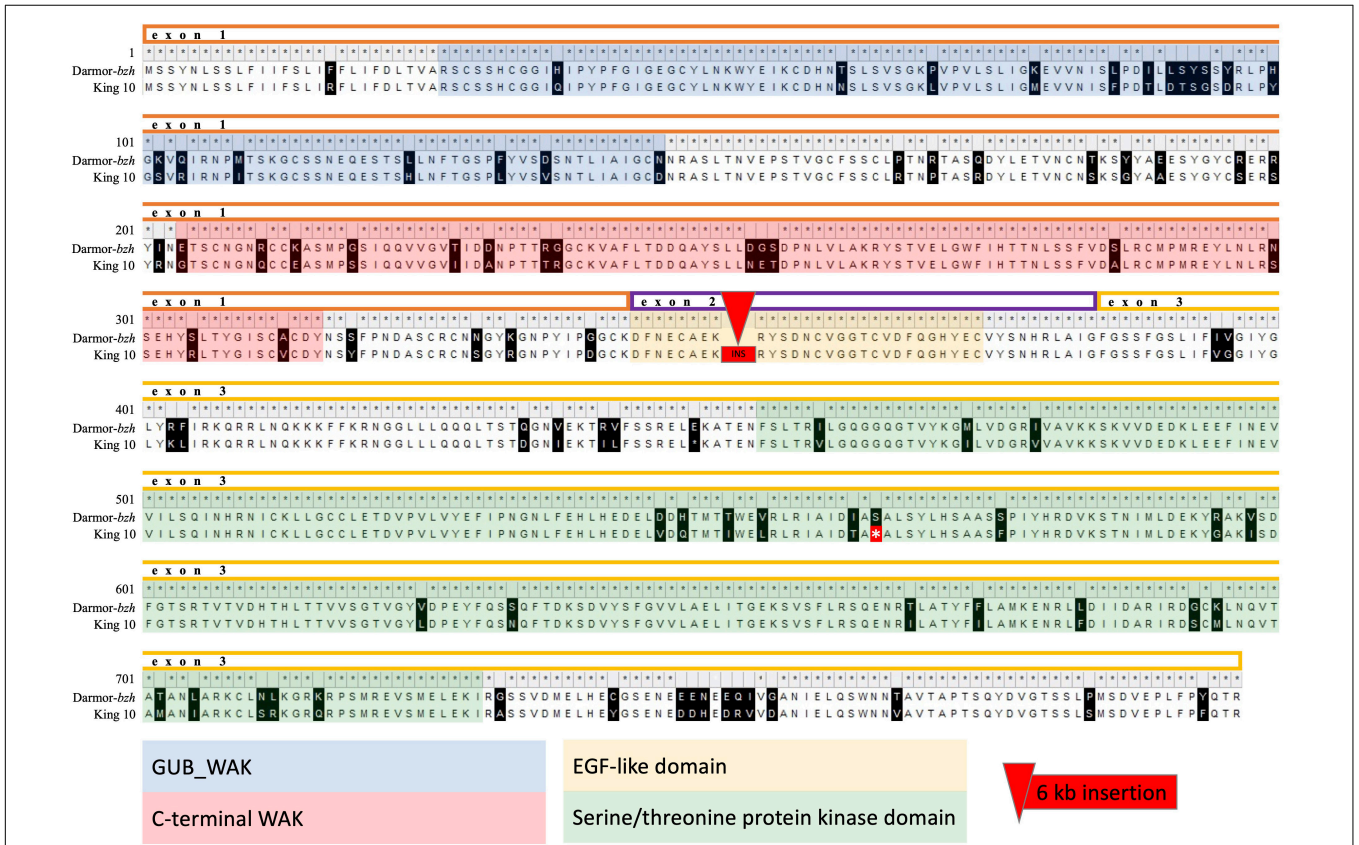



**FIGURE 2 |** Alignment of the protein sequence of King 10 against the reference Darmor-*bzh* v10. King 10 is representative for the four parental lines harboring a 6 kb insertion and 202 SNV leading to 92 non-synonymous amino acid changes. Different colors indicate exons and conserved domains. The asterisk indicates a stop codon.

partly overlap in both populations. Assuming that the same genes are involved in quantitative resistance expression in both winter oilseed rape populations, the overlap in the QTL interval might be useful to narrow the search for potential candidate genes. By considering overlapping QTL regions as high-confidence QTL intervals, we reduced the areas of interest from 888 kb to 271 kb, 998 kb to 473 kb, and 2,338 kb to 72 kb, respectively. In total, these three intervals contained 133 genes (68, 54, and 11). The number of candidate genes was reduced from 68 to 5, 54 to 2, and 11 to 1 for the three co-localizing QTL intervals for QTL A07.b304, A09.b366, and A09.b382 by filtering for GO terms associated to defense response and/or resistance (**Table 2** and **Supplementary Table 10**). *Rlm9* is localized in the overlapping QTL region of both populations, the multiparental mapping population and the diversity set tested under controlled conditions in the greenhouse and in the field, respectively.

**TABLE 2 |** Genes within the *L. maculans* resistance QTL of the multiparental population overlapping with QTL of the diversity set based on GO terms that can be associated with plant resistance and numbers of genomic variants in the multiparental population.

| Gene ID | QTL ID | Range of overlap in kb | Gene annotation | Structural variants (SV) | No. of single nucleotide variant (SNV) calls |
|---|---|---|---|---|---|
| A07p26790.1_BnaDAR | A07.b304 | 271 kb | Nuclear pore complex protein NUP96 | – | 1 |
| A07p26870.1_BnaDAR | A07.b304 | 271 kb | Cinnamoyl CoA reductase | – | 18 |
| A07p26890.1_BnaDAR | A07.b304 | 271 kb | Transcription factor WRKY | – | 17 |
| A07p27010.1_BnaDAR | A07.b304 | 271 kb | Wall-associated receptor kinase-like 10 | 6 kb insertion | 142 |
| A07p27130.1_BnaDAR | A07.b304 | 271 kb | ALA-interacting subunit | – | – |
| A09p21820.1_BnaDAR | A09.b366 | 473 kb | Protein ENHANCED DISEASE RESISTANCE 2-like | – | 96 |
| A09p21880.1_BnaDAR | A09.b366 | 473 kb | Heat shock transcription factor | – | 8 |
| A09p44190.1_BnaDAR | A09.b382 | 72 kb | UPF0183 protein | – | – |

## 4. DISCUSSION

Quantitative resistance against *L. maculans* exhibiting minor effects in *B. napus* is difficult to detect as it is frequently masked by qualitative resistances exhibiting major effects controlled by race-specific *R* genes in the host and pathogen. Pathogen-associated molecular patterns (PAMPs) are considered to initiate a broad-spectrum resistance against a pathogen species, termed PAMP-triggered immunity (PTI), whereas race-specific pathogen effectors contribute to pathogen virulence and can induce effector-triggered immunity (ETI) in the plant host on a gene-for-gene interaction model, also called qualitative resistance (Jones and Dangl, 2006). However, in the last decade it has become clear that this strict distinction between PTI and ETI might need reconsideration (Thomma et al., 2011). In addition, the extent to which PTI is associated with quantitative resistance expression also remains to be clarified (Delplace et al., 2020). ETI and PTI are both mainly involved in pathogen perception and signaling, whereas quantitative resistance goes beyond that and is controlled by numerous genes with diversified functions (Corwin and Kliebenstein, 2017). Qualitative and quantitative resistance cannot normally be clearly distinguished in field studies. The complexity of the fungal population in the field, harboring different avirulence genes, and the genetic composition of the plant population, harboring unknown minor and major resistance allele combinations, generally cause difficulties in association studies of quantitative resistance under field conditions. Thus, our strategy in this study was to identify and use a fungal *L. maculans* isolate that has no qualitative resistance *R* gene interaction with the parental oilseed rape cultivars of our multiparental population, and to use this isolate to map quantitative minor effect QTL by GWAS under controlled conditions.

Using this strategy, we identified a normal distribution of disease, indicating the exclusively quantitative inheritance of blackleg resistance in the mapping population. The absence of effective gene-for-gene interactions that can usually be observed in the *B. napus–L. maculans* pathosystem allowed us to identify genomic regions explaining only a small portion of phenotypic variation (<6%). In accordance with a previous study, we also observed high QTL-by-environment interactions, which is common for quantitatively inherited disease resistance even under controlled greenhouse conditions (Obermeier et al., 2013) and is even more common due to varying disease pressure throughout field trials (Huang et al., 2016; Larkan et al., 2016a; Kumar et al., 2018; Raman et al., 2018).

In most studies mapping quantitative blackleg resistance, different methods for disease scoring of cross sections at the crown or scoring of survival rate were used to estimate the disease severity (Huang et al., 2016; Larkan et al., 2016a; Gabur et al., 2018; Kumar et al., 2018; Raman et al., 2018; Raman R. et al., 2020). In the field trials of the present study, G2 index scoring of cross sections at the crown was applied due to the immense workload that needs to be achieved within a short period of time. However, the G2 index depends strongly on precise cuts to accurately assess the cross sections. This phenotyping method is time-saving compared to VDT scoring and provides useful and informative data from field evaluations of blackleg disease. In addition, to gain more detailed insights into the growth of the fungus, the VDT scoring method of Kutcher et al. (1993) was used for the assessments in the greenhouse trials. The high overlap of QTL results with previous studies underpins the relevance of the applied phenotyping method and further reveals that some of the identified QTL regions are crucial within international germplasm (Jestin et al., 2015; Raman et al., 2016, 2018; Kumar et al., 2018; Fikere et al., 2020; Raman H. et al., 2020; Raman R. et al., 2020; **Supplementary Table 11**).

We mapped quantitative resistance on chromosomes A09 and A07 in the multiparental population in the greenhouse. All QTL explained less than 5% of the phenotypic variation suggesting that genomic selection approaches are more suitable than marker-assisted selection for breeding toward quantitative *L. maculans* resistance in oilseed rape. Some of the QTL regions identified in the present study on chromosome A09 and A07 overlapped with previously described QTL (**Supplementary Table 11**). However, chromosome A07, in contrast to A09, is mainly known to harbor qualitative resistance genes providing race-specific resistances (Delourme et al., 2006). Although we ensured the specific assessment of quantitative resistance by selecting a fungal isolate with no gene-for-gene interaction with

the mapping population in our greenhouse trials, to our surprise a QTL explaining less than 5% of the phenotypic variance was identified in a genome region on chromosome A07 known to harbor a cluster of *R* genes (*Rlm3*, *Rlm4*, *Rlm7, and Rlm9*) involved in qualitative resistance expression. This suggests that this genome region is either a genomic hot spot where genes involved in qualitative as well as quantitative resistance are tightly clustered (linkage) or that some genes known to be involved in major qualitative resistance can also impart quantitative effects on resistance. Although it is possible that linkage exists between *R* gene clusters which are non-functional at the cotyledon stage in our population and genes involved explicitly in quantitative resistance expression in the LD block on chromosome A07, no clear candidate genes could be identified by GO analysis or polymorphism detection between the parental genotypes. The most striking polymorphism was detected in the well described major resistance gene *Rlm9*, which could suggest that this gene is also involved in adult plant resistance expression in the investigated multiparental population. In addition, *Rlm9* co-localized with an overlapping QTL region in a diversity set for field resistance in adult plants subjected to infection by an *L. maculans* population with an avirulent *AvrRlm9* gene composition. This observation supports the involvement of this candidate gene in quantitative resistance expression in these environments and populations.

This result is in accordance with previous reports that some major *Rlm*/*LepR* genes also have quantitative effects on adult plant resistance. For example, the fungal *AvrLmS-Lep2* gene-for-gene interaction with a *B. napus R* gene, *LepR2*, shows a qualitative intermediate resistance response at the cotyledon stage and partial resistance at the adult plant stage (Long et al., 2011; Dandena et al., 2019; Neik et al., 2020, preprint). The hypothesis that *Rlm9* is involved in quantitative resistance is also supported by Raman et al. (2018), who reported a quantitative resistance effect of the location harboring *Rlm9* in the greenhouse on adult plants, using a plant population segregating for *Rlm9* and an *L. maculans* isolate carrying a corresponding functional avirulence gene *AvrLm5-9* allele. That result also suggested that *Rlm9*-mediated resistance was expressed at the adult plant stage. The concept of *R* genes mediating quantitative resistance has been previously suggested and discussed in other studies that made similar observations (Chantret et al., 1999; Raman et al., 2018). A possible weak, constitutive expression of *R* genes at adult plant stage may lead to partial resistance with only minor effects. Our results suggest that further studies investigating *R* gene expression at adult plant stage or even accompanying an entire growth period could give deeper insights into these repeatedly observed findings.

Our detailed analysis of the molecular polymorphism for *Rlm9* in the parents of the mapping population also supports this hypothesis. In particular, non-synonymous amino acid changes, an inserted stop codon and especially the large insertion in *Rlm9* most likely alter the transcript or even interrupt the transcription of the gene in four of the seven parents of the mapping population. Usually, *R* genes encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (McHale et al., 2006). Wall-associated kinase (WAK) and wall-associated kinase-like (WAKL) genes are a newly discovered class of race-specific plant receptor-like kinase resistance genes involved in qualitative resistance (Larkan et al., 2020). However, although some WAKL genes have been shown to be involved in race-specific gene-for-gene interactions, for example *Rlm9* in oilseed rape and *Stb9* in wheat (Keller and Krattinger, 2018), other WAKL genes like *RFO1* (*RESISTANCE TO FUSARIUM OXYSPORUM 1*)/*WAKL22* in Arabidopsis have been found to be involved in broad-spectrum resistance against *F. oxysporum* f. sp. *matthioli* and other races (Diener and Ausubel, 2005) and against *Verticillium longisporum* (Johansson et al., 2006) in Arabidopsis, while *ZmWAK1* confers quantitative resistance to northern corn leaf blight in maize (Hurni et al., 2015). The protein structure of the WAKs and some WAKLs can be divided into an extracellular and an intracellular compartment, which are connected by a transmembrane domain. These proteins are characterized by a cytoplasmic Ser/Thr kinase domain in the cell interior and an extracellular domain that is similar to epidermal growth factor domains in vertebrates (epidermal growth factor; EGF-like domain). This occurs as a calcium-binding EGF domain (EGF-$Ca^{2+}$) and/or as an EGF2-like domain, although in some cases it is slightly degenerate (Verica and He, 2002). The exact function of these EGF-like domains is still largely unclear. However, previous studies demonstrated that EGF-like domains are involved in protein-protein interactions (Kuroda and Tanizawa, 1999). This extracellular domain is disrupted in four of the seven parents in our study by a 6 kb insertion, which might thus be expected to potentially interrupt (unknown) protein-protein-interactions and could consequently impart a quantitative impact on resistance activity. Other parts of the extracellular domain are bound to the pectin of the cell wall (Wagner and Kohorn, 2001), but can also serve as receptors for oligogalacturonides (OGs), which, among other things, arise from mechanical destruction of the pectin and act as DAMPs (damage-associated molecular patterns) to activate the plant immune system (Brutus et al., 2010). This part contains a conserved GUB_WAK domain (galacturonan-binding wall-associated receptor kinase). This suggests that WAKL genes might be involved in more broad-spectrum resistance in some pathogen-host interactions by sensing DAMPs. Hence, our results in oilseed rape suggest a possible dual function of *Rlm9*. On the one hand, *Rlm9* is expected to be involved in race-specific PTI in oilseed rape when challenged with *L. maculans* isolates carrying *AvrLm5-9*, which trigger a strong qualitative gene-for-gene resistance effect in plant genotypes with a functional interacting *Rlm9* gene as in Raman et al. (2018). However, in contrast to Raman et al. (2018), the results in our study suggest that the gene-for-gene interaction for *Rlm9* is not only expressed at the cotyledon stage, but also to a lesser extent in adult plants. In contrast to Raman et al. (2018), we used a *L. maculans* isolate which did not harbor a corresponding avirulence gene (*avrLm5-9*). Thus, even though no gene-for-gene interaction of *Rlm9* with its corresponding avirulence gene is expected in our experiment, still we found a weak expression of quantitative resistance of 5%. This indicates that *Rlm9* may have other additional features triggering quantitative resistance, for example by sensing DAMPs. Only if some functional domains are disrupted, like the EGF-like domain in four of the seven

genotypes in our study, might this weak quantitative resistance effect also be lost. All in all, the results of the present study confer with previous studies that observed *R* gene mediated resistance under controlled and field conditions at adult plant stages in *B. napus* (Delourme et al., 2004; Raman et al., 2012). Based on these collective observations, we hypothesize that the role of *Rlm9* in this interaction deserves more detailed functional analysis in future.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: PRJNA751459).

## AUTHOR CONTRIBUTIONS

CO and RS conceived the idea and sourced the funding. PV, HC, and CO developed the methodology. PV generated the genetic and field data. DA and BK generated the greenhouse data. PV and CO performed data curation. PV, HC, and HL analyzed the sequence and SV data. PV, SW, LE, and IG performed the quantitative genetic analysis. PV, RS, and CO drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

PV and CO received funding from the German Federal Ministry of Education and Research (BMBF) within the project PhomaDur (Grant Number BLE: 2818205115). This work was also supported by the BMBF-funded de.NBI Cloud within the German Network for Bioinformatics Infrastructure (de.NBI) (031A537B, 031A533A, 031A538A, 031A533B, 031A535A, 031A537C, 031A534A, and 031A532B).

## ACKNOWLEDGMENTS

We thank Regina Illgner, Jutta Schaper, and Anita Popovska for excellent technical help. We also thank all German breeding companies organized in the GFPi (Gemeinschaft zur Förderung von Pflanzeninnovation e. V.) for production of the elite NAM population, for performing field trials, and for providing phenotyping data.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.749491/full#supplementary-material

**Supplementary Figure 1 |** Phenotypic distribution of VDT values from greenhouse screenings for the entire mapping population **(A)** and the individual subfamilies: Adriana × Lorenz **(B)**, Lorenz × Alpaga **(C)**, Lorenz × DK Cabernet **(D)**, Lorenz × Galileo **(E)**, JN × Lorenz **(F)**, and King 10 × Lorenz **(G)**.

## REFERENCES

Aubertot, J.-N., Schott, J.-J., Penaud, A., Brun, H., and Doré, T. (2004). Methods for sampling and assessment in relation to the spatial pattern of phoma stem canker (*Leptosphaeria maculans*) in oilseed rape. *Eur. J. Plant Pathol.* 110, 183–192. doi: 10.1023/B:EJPP.0000015359.61910.3b

Aulchenko, Y. S., Koning, D.-J. D., and Haley, C. (2007). Genomewide Rapid Association using mixed model and regression: a fast and simple method for genomewide pedigree-based quantitative trait Loci Association analysis. *Genetics* 177, 577–585. doi: 10.1534/genetics.107.075614

Balesdent, M. H., Attard, A., Ansan-Melayah, D., Delourme, R., Renard, M., and Rouxel, T. (2001). Genetic Control and host range of avirulence toward *Brassica napus* cultivars quinta and jet neuf in *Leptosphaeria maculans*. *Phytopathology* 91, 70–76. doi: 10.1094/PHYTO.2001.91.1.70

Balesdent, M. H., Attard, A., Kühn, M. L., and Rouxel, T. (2002). New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. *Phytopathology* 92, 1122–1133. doi: 10.1094/PHYTO.2002.92.10.1122

Balesdent, M. H., Barbetti, M. J., Li, H., Sivasithamparam, K., Gout, L., and Rouxel, T. (2005). Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathology* 95, 1061–1071. doi: 10.1094/PHYTO-95-1061

Balesdent, M.-H., Louvard, K., Pinochet, X., and Rouxel, T. (2006). A large-scale survey of races of *Leptosphaeria maculans* occurring on oilseed rape in France. *Eur. J. Plant Pathol.* 114, 53–65. doi: 10.1007/s10658-005-2104-0

Bayer, P. E., Hurgobin, B., Golicz, A. A., Chan, C.-K. K., Yuan, Y., Lee, H., et al. (2017). Assembly and comparison of two closely related *Brassica napus* genomes. *Plant Biotechnol. J.* 15, 1602–1610. doi: 10.1111/pbi.12742

Brun, H., Chèvre, A.-M., Fitt, B. D. L., Powers, S., Besnard, A.-L., Ermel, M., et al. (2010). Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytol.* 185, 285–299. doi: 10.1111/j.1469-8137.2009.03049.x

Brutus, A., Sicilia, F., Macone, A., Cervone, F., and de Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9452–9457. doi: 10.1073/pnas.1000675107

Bus, A., Körber, N., Snowdon, R. J., and Stich, B. (2011). Patterns of molecular variation in a species-wide germplasm set of *Brassica napus*. *Theor. Appl. Genet.* 123, 1413–1423. doi: 10.1007/s00122-011-1676-7

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I. A. P., Tang, H., Wang, X., et al. (2014). Early allopolyploid evolution in the post-neolithic *Brassica napus* oilseed genome. *Science* 345, 950–953. doi: 10.1126/science.1253435

Chantret, N., Pavoine, M. T., and Doussinault, G. (1999). The race-specific resistance gene to powdery mildew, MlRE, has a residual effect on adult plant resistance of winter wheat line RE714. *Phytopathology* 89, 533–539. doi: 10.1094/PHYTO.1999.89.7.533

Chawla, H. S., Lee, H., Gabur, I., Vollrath, P., Tamilselvan-Nattar-Amutha, S., Obermeier, C., et al. (2020). Long-read sequencing reveals widespread intragenic structural variants in a recent allopolyploid crop plant. *Plant Biotechnol. J.* 19, 240–250. doi: 10.1111/pbi.13456

Cheng, C. Y., Krishnakumar, V., Chan, A. P., Thibaud-Nissen, F., Schobel, S., and Town, C. D. (2017). Araport11: a complete reannotation of the *Arabidopsis thaliana* reference genome. *Plant J.* 89, 789–804. doi: 10.1111/tpj.13415

Corwin, J. A., and Kliebenstein, D. J. (2017). Quantitative resistance: more than just perception of a pathogen. *Plant Cell* 29, 655–665. doi: 10.1105/tpc.16.00915

Dandena, H. B., Zhang, Q., Zhou, T., Hirani, A. H., Liu, Z., Fernando, D. W. G., et al. (2019). Analysis of quantitative adult plant resistance to blackleg in *Brassica napus*. *Mol. Breed.* 39:124. doi: 10.1007/s11032-019-1035-y

Delourme, R., Bousset, L., Ermel, M., Duffé, P., Besnard, A. L., Marquer, B., et al. (2014). Quantitative resistance affects the speed of frequency increase but not

the diversity of the virulence alleles overcoming a major resistance gene to *Leptosphaeria maculans* in oilseed rape. *Infect. Genet. Evol.* 27, 490–499. doi: 10.1016/j.meegid.2013.12.019

Delourme, R., Chèvre, A. M., Brun, H., Rouxel, T., Balesdent, M. H., Dias, J. S., et al. (2006). Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *Eur. J. Plant Pathol.* 114, 41–52. doi: 10.1007/s10658-005-2108-9

Delourme, R., Pilet-Nayel, M.-L., Archipiano, M., Horvais, R., Tanguy, X., Rouxel, T., et al. (2004). A cluster of major specific resistance genes to *Leptosphaeria maculans* in *Brassica napus*. *Phytopathology* 94, 578–583. doi: 10.1094/phyto.2004.94.6.578

Delplace, F., Huard-Chauveau, C., Dubiella, U., Khafif, M., Alvarez, E., Langin, G., et al. (2020). Robustness of plant quantitative disease resistance is provided by a decentralized immune network. *Proc. Natl. Acad. Sci. U.S.A.* 117, 18099–18109. doi: 10.1073/pnas.2000078117

Diener, A. C., and Ausubel, F. M. (2005). RESISTANCE TO FUSARIUM OXYSPORUM 1, a dominant *Arabidopsis* disease-resistance gene, is not race specific. *Genetics* 171, 305–321. doi: 10.1534/genetics.105.042218

Elliott, V. L., Marcroft, S. J., Howlett, B. J., and van de Wouw, A. P. (2016). Gene-for-gene resistance is expressed in cotyledons, leaves and pods, but not during late stages of stem colonization in the *Leptosphaeria maculans*-*Brassica napus* pathosystem. *Plant Breed.* 135, 200–207. doi: 10.1111/pbr.12343

Fikere, M., Barbulescu, D. M., Malmberg, M. M., Spangenberg, G. C., Cogan, N. O. I., and Daetwyler, H. D. (2020). Meta-analysis of GWAS in canola blackleg (*Leptosphaeria maculans*) disease traits demonstrates increased power from imputed whole-genome sequence. *Sci. Rep.* 10:14300. doi: 10.1038/s41598-020-71274-6

Fitt, B. D. L., Brun, H., Barbetti, M. J., and Rimmer, S. R. (2006). World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *Eur. J. Plant Pathol.* 114, 3–15. doi: 10.1007/s10658-005-2233-5

Gabur, I., Chawla, H. S., Liu, X., Kumar, V., Faure, S., Tiedemann, A. V., et al. (2018). Finding invisible quantitative trait loci with missing data. *Plant Biotechnol. J.* 16, 2102–2112. doi: 10.1111/pbi.12942

Gabur, I., Chawla, H. S., Lopisso, D. T., Tiedemann, A. V., Snowdon, R. J., and Obermeier, C. (2020). Gene presence-absence variation associates with quantitative *Verticillium longisporum* disease resistance in *Brassica napus*. *Sci. Rep.* 10:4131. doi: 10.1038/s41598-020-61228-3

Huang, Y. J., Jestin, C., Welham, S. J., King, G. J., Manzanares-Dauleux, M. J., Fitt, B. D. L., et al. (2016). Identification of environmentally stable QTL for resistance against *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *Theor. Appl. Genet.* 129, 169–180. doi: 10.1007/s00122-015-2620-z

Huang, Y. J., Pirie, E. J., Evans, N., Delourme, R., King, G. J., and Fitt, B. D. L. (2009). Quantitative resistance to symptomless growth of *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape). *Plant Pathol.* 58, 314–323. doi: 10.1111/j.1365-3059.2008.01957.x

Hurgobin, B., Golicz, A. A., Bayer, P. E., Chan, C.-K. K., Tirnaz, S., Dolatabadian, A., et al. (2018). Homoeologous exchange is a major cause of gene presence/absence variation in the amphidiploid *Brassica napus*. *Plant Biotechnol. J.* 16, 1265–1274. doi: 10.1111/pbi.12867

Hurni, S., Scheuermann, D., Krattinger, S. G., Kessel, B., Wicker, T., Herren, G., et al. (2015). The maize disease resistance gene Htn1 against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8780–8785. doi: 10.1073/pnas.1502522112

Jestin, C., Bardol, N., Lodé, M., Duffé, P., Domin, C., Vallée, P., et al. (2015). Connected populations for detecting quantitative resistance factors to phoma stem canker in oilseed rape (*Brassica napus* L.). *Mol. Breed.* 35:167. doi: 10.1007/s11032-015-0356-8

Johansson, A., Staal, J., and Dixelius, C. (2006). Early responses in the *Arabidopsis*-*Verticillium longisporum* pathosystem are dependent on NDR1, JA- and ET-associated signals via cytosolic NPR1 and RFO1. *Mol. Plant Microbe Interact.* 19, 958–969. doi: 10.1094/MPMI-19-0958

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Keller, B., and Krattinger, S. G. (2018). A new player in race-specific resistance. *Nat. Plants* 4, 197–198. doi: 10.1038/s41477-018-0126-9

Kumar, V., Paillard, S., Fopa Fomeju, B., Falentin, C., Deniot, G., Baron, C., et al. (2018). Multi-year linkage and association mapping confirm the high number of genomic regions involved in oilseed rape quantitative resistance to blackleg. *Theor. Appl. Genet.* 131, 1627–1643. doi: 10.1007/s00122-018-3103-9

Kuroda, S., and Tanizawa, K. (1999). Involvement of epidermal growth factor-like domain of NELL proteins in the novel protein-protein interaction with protein kinase C. *Biochem. Biophys. Res. Commun.* 265, 752–757. doi: 10.1006/bbrc.1999.1753

Kutcher, H. R., van den Berg, C. G. J., and Rimmer, S. R. (1993). Variation in pathogenicity of *Leptosphaeria maculans* on *Brassica* spp. based on cotyledon and stem reactions. *Can. J. Plant Pathol.* 15, 253–258. doi: 10.1080/07060669309501920

Kuznetsova, A., Brockhoff, P. B., and Christensen, R. H. B. (2017). lmertest package: tests in linear mixed effects models. *J. Stat. Softw.* 82, 1–26. doi: 10.18637/jss.v082.i13

Lancashire, P. D., Bleiholder, H., van den Boom, T., Langelüddeke, P., Stauss, R., Elfriede, W., et al. (1991). A uniform decimal code for growth stages of crops and weeds. *Ann. Appl. Biol.* 1991, 561–601. doi: 10.1111/j.1744-7348.1991.tb04895.x

Larkan, N. J., Ma, L., Haddadi, P., Buchwaldt, M., Parkin, I. A. P., Djavaheri, M., et al. (2020). The *Brassica napus* wall-associated kinase-like (WAKL) gene Rlm9 provides race-specific blackleg resistance. *Plant J.* 104, 892–900. doi: 10.1111/tpj.14966

Larkan, N. J., Raman, H., Lydiate, D. J., Robinson, S. J., Yu, F., Barbulescu, D. M., et al. (2016a). Multi-environment QTL studies suggest a role for cysteine-rich protein kinase genes in quantitative resistance to blackleg disease in *Brassica napus*. *BMC Plant Biol.* 16:183. doi: 10.1186/s12870-016-0877-2

Larkan, N. J., Yu, F., Lydiate, D. J., Rimmer, S. R., and Borhan, M. H. (2016b). Single R gene introgression lines for accurate dissection of the *Brassica*–*Leptosphaeria* pathosystem. *Front. Plant Sci.* 7:1771. doi: 10.3389/fpls.2016.01771

Lenth, R. V. (2016). Least-squares means: the R package lsmeans. *J. Stat. Softw.* 69, 1–33. doi: 10.18637/jss.v069.i01

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Long, Y., Wang, Z., Sun, Z., Fernando, D. W. G., McVetty, P. B. E., and Li, G. (2011). Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola cultivar 'Surpass 400'. *Theor. Appl. Genet.* 122, 1223–1231. doi: 10.1007/s00122-010-1526-z

Luo, R., Wong, C.-L., Wong, Y.-S., Tang, C.-I., Liu, C.-M., Leung, C.-M., et al. (2020). Exploring the limit of using a deep neural network on pileup data for germline variant calling. *Nat. Mach. Intell.* 2, 220–227. doi: 10.1038/s42256-020-0167-4

Marcroft, S. J., Elliott, V. L., Cozijnsen, A. J., Salisbury, P. A., Howlett, B. J., and van de Wouw, A. P. (2012). Identifying resistance genes to *Leptosphaeria maculans* in Australian *Brassica napus* cultivars based on reactions to isolates with known avirulence genotypes. *Crop Pasture Sci.* 63, 338–350. doi: 10.1071/CP11341

McHale, L., Tan, X., Koehl, P., and Michelmore, R. W. (2006). Plant NBS-LRR proteins: adaptable guards. *Genome Biol.* 7:212. doi: 10.1186/gb-2006-7-4-212

Neik, T. X., Ghanbarnia, K., Ollivier, B., Scheben, A., Severn-Ellis, A., Larkan, N. J., et al. (2020). Two independent approaches converge to the cloning of a new *Leptosphaeria maculans* avirulence effector gene, AvrLmS-Lep2. *bioRxiv* [Preprint]. doi: 10.1101/2020.10.02.322479

Obermeier, C., Hossain, M. A., Snowdon, R., Knüfer, J., von Tiedemann, A., and Friedt, W. (2013). Genetic analysis of phenylpropanoid metabolites associated with resistance against *Verticillium longisporum* in *Brassica napus*. *Mol. Breed.* 31, 347–361. doi: 10.1007/s11032-012-9794-8

Parkin, I. A., Sharpe, A. G., Keith, D. J., and Lydiate, D. J. (1995). Identification of the A and C genomes of amphidiploid *Brassica napus* (oilseed rape). *Genome* 38, 1122–1131. doi: 10.1139/g95-149

Pilet, M. L., Delourme, R., Foisset, N., and Renard, M. (1998). Identification of QTL involved in field resistance to light leaf spot (*Pyrenopeziza brassicae*) and blackleg resistance (*Leptosphaeria maculans*) in winter rapeseed (*Brassica napus* L.). *Theor. Appl. Genet.* 97, 398–406. doi: 10.1007/s001220050909

Pilet-Nayel, M.-L., Moury, B., Caffier, V., Montarry, J., Kerlan, M.-C., Fournet, S., et al. (2017). Quantitative resistance to plant pathogens in pyramiding strategies for durable crop protection. *Front. Plant Sci.* 8:1838. doi: 10.3389/fpls.2017.01838

Raman, H., McVittie, B., Pirathiban, R., Raman, R., Zhang, Y., Barbulescu, D. M., et al. (2020). Genome-Wide Association mapping identifies novel Loci for

quantitative resistance to blackleg disease in canola. *Front. Plant Sci.* 11:1184. doi: 10.3389/fpls.2020.01184

Raman, H., Raman, R., Coombes, N., Song, J., Diffey, S., Kilian, A., et al. (2016). Genome-wide Association study identifies new loci for resistance to *Leptosphaeria maculans* in canola. *Front. Plant Sci.* 7:1513. doi: 10.3389/fpls.2016.01513

Raman, H., Raman, R., Diffey, S., Qiu, Y., McVittie, B., Barbulescu, D. M., et al. (2018). Stable quantitative resistance loci to blackleg disease in canola (*Brassica napus* L.) over continents. *Front. Plant Sci.* 9:1622. doi: 10.3389/fpls.2018.01622

Raman, R., Diffey, S., Barbulescu, D. M., Coombes, N., Luckett, D., Salisbury, P., et al. (2020). Genetic and physical mapping of loci for resistance to blackleg disease in canola (*Brassica napus* L.). *Sci. Rep.* 10:4416. doi: 10.1038/s41598-020-61211-y

Raman, R., Taylor, B., Marcroft, S., Stiller, J., Eckermann, P., Coombes, N., et al. (2012). Molecular mapping of qualitative and quantitative loci for resistance to *Leptosphaeria maculans* causing blackleg disease in canola (*Brassica napus* L.). *Theor. Appl. Genet.* 125, 405–418. doi: 10.1007/s00122-012-1842-6

Rimmer, S. R., and van den Berg, C. G. J. (1992). Resistance of oilseed *Brassica* spp. to blackleg caused by *Leptosphaeria maculans*. *Can. J. Plant Pathol.* 14, 56–66. doi: 10.1080/07060669209500906

Rousseau-Gueutin, M., Belser, C., Da Silva, C., Richard, G., Istace, B., Cruaud, C., et al. (2020). Long-read assembly of the *Brassica napus* reference genome Darmor-bzh. *Gigascience* 9, 1–16. doi: 10.1093/gigascience/giaa137

Rouxel, T., Penaud, A., Pinochet, X., Brun, H., Gout, L., Delourme, R., et al. (2003). A 10-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the Rlm1 resistance gene of oilseed rape. *Eur. J. Plant Pathol.* 109, 871–881. doi: 10.1023/A:1026189225466

Samans, B., Chalhoub, B., and Snowdon, R. J. (2017). Surviving a genome collision: genomic signatures of allopolyploidization in the recent crop species *Brassica napus*. *Plant Genome* 10, 1–15. doi: 10.3835/plantgenome2017.02.0013

Sedlazeck, F. J., Rescheneder, P., Smolka, M., Fang, H., Nattestad, M., Haeseler, A. V., et al. (2018). Accurate detection of complex structural variations using single-molecule sequencing. *Nat. Methods* 15, 461–468. doi: 10.1038/s41592-018-0001-7

Sprague, S. J., Balesdent, M.-H., Brun, H., Hayden, H. L., Marcroft, S. J., Pinochet, X., et al. (2006). Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. *Eur. J. Plant Pathol.* 114, 33–40. doi: 10.1007/s10658-005-3683-5

St Clair, D. A. (2010). Quantitative disease resistance and quantitative resistance loci in breeding. *Annu. Rev. Phytopathol.* 48, 247–268. doi: 10.1146/annurev-phyto-080508-081904

Stein, A., Coriton, O., Rousseau-Gueutin, M., Samans, B., Schiessl, S. V., Obermeier, C., et al. (2017). Mapping of homoeologous chromosome exchanges influencing quantitative trait variation in *Brassica napus*. *Plant Biotechnol. J.* 15, 1478–1489. doi: 10.1111/pbi.12732

Szadkowski, E., Eber, F., Huteau, V., Lodé, M., Huneau, C., Belcram, H., et al. (2010). The first meiosis of resynthesized *Brassica napus*, a genome blender. *New Phytol.* 186, 102–112. doi: 10.1111/j.1469-8137.2010.03182.x

Thomma, B., Nürnberger, T., and Joosten, M. H. A. J. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *Plant Cell* 23, 4–15. doi: 10.1105/tpc.110.082602

Nagaharu, U. (1935). Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. *Jpn. J. Bot.* 7, 389–453.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J. A. M. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res.* 35, W71–W74. doi: 10.1093/nar/gkm306

van de Wouw, A. P., Howlett, B. J., and Idnurm, A. (2017). Changes in allele frequencies of avirulence genes in the blackleg fungus, *Leptosphaeria maculans*, over two decades in Australia. *Crop Pasture Sci.* 69, 20. doi: 10.1071/CP16411

Verica, J. A., and He, Z. (2002). The cell Wall-Associated Kinase (WAK) and WAK-like kinase gene family. *Plant Physiol.* 129, 455–459.

Wagner, T. A., and Kohorn, B. D. (2001). Wall-Associated Ainases are expressed throughout plant development and are required for cell expansion. *Plant Cell* 13, 303–318. doi: 10.1105/tpc.13.2.303

West, J. S., Kharbanda, P. D., Barbetti, M. J., and Fitt, B. D. L. (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathol.* 50, 10–27. doi: 10.1046/j.1365-3059.2001.00546.x

Winter, M., and Koopmann, B. (2016). Race spectra of *Leptosphaeria maculans*, the causal agent of blackleg disease of oilseed rape, in different geographic regions in northern Germany. *Eur. J. Plant Pathol.* 145, 629–641. doi: 10.1007/s10658-016-0932-8

Zhang, X., Peng, G., Kutcher, H. R., Balesdent, M.-H., Delourme, R., and Fernando, W. G. D. (2016). Breakdown of Rlm3 resistance in the *Brassica napus*–*Leptosphaeria maculans* pathosystem in western Canada. *Eur. J. Plant Pathol.* 145, 659–674. doi: 10.1007/s10658-015-0819-0

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Chromosome Res (2019) 27:221–236
https://doi.org/10.1007/s10577-019-09612-2

ORIGINAL ARTICLE

# Hybrids between *Brassica napus* and *B. nigra* show frequent pairing between the B and A/C genomes and resistance to blackleg

**Roman Gaebelein · Dima Alnajar · Birger Koopmann · Annaliese S. Mason**

Received: 12 March 2019 / Revised: 24 June 2019 / Accepted: 27 June 2019 / Published online: 6 July 2019


**Abstract** High frequencies of homoeologous and even non-homologous chromosome recombination in *Brassica* hybrids can transfer useful traits between genomes, but also destabilise synthetic allopolyploids. We produced triploid hybrids ($2n = 3x =$ ABC) from the cross *B. napus* (rapeseed, $2n = 4x =$ AACC) × *B. nigra* (black mustard, $2n = 2x =$ BB) by embryo rescue and allohexaploid hybrids ($2n = 6x =$ AABBCC = 54) by chromosome doubling of the triploids. These hybrids demonstrated resistance to blackleg disease (causal agent: *Leptosphaeria maculans*) inherited from their *B. nigra* parent. In order to assess the possibility of transfer of this resistance between the B genome and the A and C subgenomes of *B. napus*, as well as to assess the genomic stability of allohexaploids from the cross *B. napus* × *B. nigra*, frequencies of non-homologous chromosome pairing in these hybrids were assessed using classical cytogenetics and genomic in-situ hybridization. Meiosis was highly irregular, and non-homologous chromosome pairing between the B genome and the A/C genomes was common in both triploid hybrids (observed in 38% of pollen mother cells) and allohexaploid hybrids (observed in 15% of pollen mother cells). Our results suggest that introgression of blackleg resistance from the B genome into the A or C genomes should be possible, but that allohexaploids from this genome combination are likely unstable.

**Keywords** *Brassica* · Interspecific hybridization · Cytogenetics · Meiotic stability · *Leptosphaeria maculans* · Introgression breeding

**Abbreviations**

| | |
|---|---|
| ANOVA | Analysis of variance |
| BAC-FISH | Bacterial artificial chromosome fluorescent in-situ hybridization |
| CMS | Cytoplasmic male sterility |
| DNA | Deoxyribonucleic acid |
| FDR | False discovery rate |
| GISH | Genomic in-situ hybridization |
| PMC | Pollen mother cell |
| VDT | Volume of diseased tissue in scoring blackleg disease infection of rapeseed |
| HR | The radius of healthy tissue in the cross section of the stem in scoring blackleg disease infection of rapeseed |
| G | Girdling of the stem according to a 0–9 point scale for scoring blackleg disease infection of rapeseed |

Responsible Editor: Hans de Jong.

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s10577-019-09612-2) contains supplementary material, which is available to authorized users.

R. Gaebelein · A. S. Mason (✉)
Department of Plant Breeding, Justus Liebig University, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany
e-mail: Annaliese.Mason@agrar.uni-giessen.de

D. Alnajar · B. Koopmann
Plant Pathology and Crop Protection Division, Department of Crop Sciences, Georg-August University Göttingen, Grisebachstraße 6, 37077 Göttingen, Germany

| | |
|---|---|
| L | Lesion length according to a 0–9 point scale for scoring blackleg disease infection of rapeseed |
| P | Profundity of necrosis according to a 0–9 point scale for scoring blackleg disease infection of rapeseed |

## 1. Introduction

Allopolyploid speciation, where the genomes of two separate species combine to form a new species, is especially common in plant evolution (Soltis et al. 2015). Many domesticated crop species are also allopolyploid, such as wheat, cotton and rapeseed, the latter of which is the second most important oil crop worldwide (USDA 2018). The development of artificial allopolyploid crops has great potential to benefit agriculture if these crops can be developed to the point where they show increased biomass or yield through inter-subgenomic heterosis, better resistance against pests and diseases or increased environmental adaptability (Mason and Batley 2015). Additionally, interspecific hybridisation is frequently used to improve crop species by transferring useful agronomic traits between species, such as resistance against fungal pathogens (Hajjar and Hodgkin 2007), and for the development of cytoplasmic male sterility systems (CMS) important for hybrid breeding. The most widely applied CMS system in *Brassica* was developed based on the transfer of the *Rfo* fertility restorer gene from *Raphanus sativus* to *Brassica napus* (Delourme et al. 1998). Introgression breeding also successfully transferred powdery mildew resistance from Asian and American grape species to *Vitis vinifera*, the domesticated grapevine (Qiu et al. 2015). Similarly, disease resistance against chestnut blight and ink disease have been transferred to the European chestnut (*Castanea sativa*) by hybridization with Asian chestnut species (Pereira-Lorenzo et al. 2016). Rapeseed production could also benefit from resistance against major fungal pathogen *Leptophaeria maculans* introduced from closely related species (Chèvre et al. 2008).

Rapeseed (*B. napus*) is part of a remarkable complex of related diploid and tetraploid species discovered by Morinaga in 1934 and later popularized as U's Triangle (U N 1935). The three diploid species *B. rapa* ($2n = 2x = AA = 20$ chromosomes; Asian cabbages, turnip), *B. nigra* ($2n = 2x = BB = 16$ chromosomes; black mustard) and *B. oleracea* ($2n = 2x = CC = 18$ chromosomes; European cabbages, cauliflower, broccoli) combined to give rise to three allotetraploid species, *B. napus* ($2n = 4x = AACC = 38$ chromosomes; rapeseed, swede), *B. juncea* ($2n = 4x = AABB = 36$ chromosomes; Indian mustard, leaf mustard) and *B. carinata* ($2n = 4x = BBCC = 34$ chromosomes; Ethiopian mustard). The interrelationship of these species suggests that the three subgenomes (A, B and C) can coexist in a single allohexaploid hybrid ($2n = 6x = AABBCC = 54$), although no natural allohexaploid hybrid has been observed. The first artificial production of such a hybrid was reported by Howard in 1942. The simplest way to produce these allohexaploid hybrids is by crossing a tetraploid and diploid species to produce a karyotype combining the three subgenomes. The least exploited method until now is to cross *B. napus* (AACC) and *B. nigra* (BB), resulting in the formation of a triploid hybrid (ABC) from which a hexaploid hybrid (AABBCC) can be produced through chromosome doubling (Gaebelein and Mason 2018). The development of allohexaploid hybrids from *B. napus* and *B. nigra* is reported to be difficult, with low pollination success rates and frequent matromorphy amongst putative hybrids (Pradhan et al. 2010a), as well as low fertility of confirmed hybrids. Chromosome doubling of the triploid to produce allohexaploid hybrids was reported as unsuccessful by one study (Jahier et al. 1989) and successful only at low frequencies in another two studies (Struss et al. 1991; Pradhan et al. 2010a). Previous studies were largely inconclusive in analysis of meiotic stability in early generations of allohexaploid *B. napus* × *B. nigra* hybrids. Meiotic pairing configuration was reported by only one study for a single hybrid plant (Struss et al. 1991). Overall, it is unknown what degree of chromosome pairing is expected to occur between the B genome and the A and C genomes in *B. napus* × *B. nigra* hybrids. Therefore, the chance of success in introgressing traits from the B genome to the A and C genomes in *Brassica* is also unknown, as is the chance of producing stable allohexaploid crop types from this genome combination (Gaebelein and Mason 2018).

Strong resistance against *L. maculans* has been identified in all species containing the B subgenome (Delourme et al. 2006), but only resistance from *B. juncea* (*Rlm6*) has so far been exploited for introgression into major oilseed crop *B. napus* (Chèvre et al. 2008). As yet, no new cultivar carrying this resistance

from *B. juncea* has been released (Rashid et al. 2018; Huang et al. 2018). Resistance against *L. maculans* conferred by single genes is frequently overcome by fast mutation of the pathogen (Rouxel and Balesdent 2017). Hence, new resistance genes from other B-genome species introgressed into *B. napus* could help to provide resistant rapeseed cultivars for the future.

We aimed to evaluate the potential value of *B. napus* × *B. nigra* triploids and allohexaploids for breeding of *L. maculans* resistant-rapeseed and for the potential to establish a new allohexaploid *Brassica* crop. Both hinge on meiotic pairing in the first generation hybrids. A stable allohexaploid crop would require regular pairing between homologous chromosomes, without interactions between non-homologous chromosomes (Mason et al. 2012). Except for one allohexaploid hybrid produced by crossing a particular genotype of *B. rapa* with *B. carinata* (Gupta et al. 2016), all allohexaploid hybrids produced to date show varying frequencies of non-homologous pairing, resulting in low fertility and genome structural variation (Gaebelein and Mason 2018). Since *B. napus* generally prevents homoeologous pairing between the highly similar A and C subgenomes, we hypothesized that genetic or genomic factors transferred from *B. napus* could also stabilize meiosis in the allohexaploid hybrid. However, successful transfer of resistance genes from the *B. nigra* B genome into the A and C subgenomes of *B. napus* is only possible if non-homologous (putatively homoeologous) chromosome pairing and recombination occurs. With this in mind, we produced interspecific hybrids between *B. napus* and *B. nigra*, assessed meiotic chromosome pairing in both triploids and allohexaploids and determined if blackleg resistance from *B. nigra* was successfully expressed in these interspecific hybrid types.

## 2. Material and methods

### Experimental material

Crosses between *Brassica napus* and *B. nigra* were made using spring-type *B. napus* accessions 'Boomer' and 'Monty_28DH' (provided by Canola Breeders Western Australia) and semi-winter type 'Ningyou7' (provided by Huazhong Agricultural University, China). Further crosses were made using the winter-type *B. napus* varieties 'MSL 007 C' (provided by NPZ Innovations GmbH, Hohenlieth, Germany), a male sterile cultivar, and *B. napus* 'Express 617' (a cultivar produced by NPZ GmbH, Hohenlieth, Germany and provided by Christian Obermeier, Justus-Liebig University Giessen, Germany). The *B. nigra* accessions NGB 21858.1 and NGB 23253.2 were sourced from the Nordgen germplasm bank, and are hereafter referred to as "IX7" and "IX13" respectively; cultivar 'Junius' was provided by Anne-Marie Chèvre, INRA Rennes, France. All *B. nigra* were used as pollen donors in the crosses with *B. napus* as the female parents.

### Plant cultivation

Seeds were sown in quickpot trays in a potting mix containing peat, bark, perlite and clay (Fruhstorfer Pflanzerde Typ N, Fruhstrofer Erde Gmbh, Germany). Plants were potted in pots of 1 l volume when the second or third set of leaves emerged. Plants were watered as required. Winter-type *B. napus* cultivars were vernalized at 5 °C for 8–10 weeks to induce flowering. Throughout the growing period, the insecticides Vertimec, Neemazal, Calypso, Pirimus and Conserve were applied to reduce insect damage from thrips and aphids. Powdery mildew was treated with Proline and Corbel. During the growing period, all plants were fertilized once per week with Wuxal Super, a liquid fertilizer containing macro and micro nutrients. Plants were kept at a minimum temperature of 18 °C during the day and 16 °C at night. Supplementary lighting was used to provide a 16-h photoperiod.

In vitro grown plants were transferred into quickpot trays, covered with a transparent plastic hood and subsequently adapted to normal greenhouse condition humidity. After 2 to 4 weeks, plants were re-potted into small pots (350 ml). Triploid $F_1$ hybrid plants and in vitro colchicine-treated hybrids were vernalized as described previously and planted in 1 l pots after vernalization.

### Pollination and embryo rescue

*Brassica napus* was used as the female parent in all cross combinations performed. In cross combinations using the male-sterile cultivar 'MSL 007 C', inflorescences were protected from pollen contamination by covering with micro-perforated transparent plastic bags and flowers were pollinated with *B. nigra* pollen in intervals of 2–3 days. Crosses with male-fertile

*B. napus* cultivars were performed by pollination of flowers in the morning the day after emasculation, following which pollinated flowers were covered using micro-perforated plastic bags.

Siliques showing swollen sections indicating developing ovules were harvested 12 days after pollination after ensuring the presence of 'torpedo stage' embryos in the ovules. Embryo rescue was performed following the protocol in Jahier (1992) using hormone-free tissue culture medium for embryo germination. Embryos were not dissected out of ovules but ovules were opened to expose embryos to tissue culture medium by piercing with pointed tweezers. Germinated embryos and small plants were transferred to hormone-free MS medium every 4 to 6 weeks for further development.

### Colchicine treatment

Plants were multiplied by subculture of nodal segments to produce multiple clones of plants grown from the same embryo. Ten $F_1$ triploid plants from four of the five cross combinations were chosen for in vitro colchicine treatment. For each hybrid, three to 12 rooted clones were cultured on MS medium supplemented with 100 mg/l colchicine, 1.5 mg/l 6-benzylaminopurine (BAP) and 0.25 mg/l 1-naphthaleneacetic acid (Ge et al. 2009). After 10 days, the colchicine medium was removed from the plants together with the roots and plants were transferred to normal MS medium to recover. After 3 to 4 weeks, plants were planted out in the greenhouse.

Additional plants were treated with colchicine by placing rooted cuttings from triploid hybrids into 0.2% colchicine solution supplemented with 1% dimethyl sulfoxide. After 24 h, plants were washed and planted into the previously described potting mix.

### Flow cytometry

Approximately 0.5 $cm^2$ of fresh leaf material from the triploid $F_1$ hybrids and colchicine-treated hybrids was chopped using a razor blade in CyStain® UV Precise P kit extraction buffer. The suspension was filtered through a 30-μm CellTrics® disposable filter. Staining buffer was added to the supernatant and the suspension was allowed to incubate for 45 s. Ploidy was analyzed using a Partec Ploidy Analyzer. A minimum of 400 nuclei giving peaks with less than 5% coefficient of variance were analyzed and the results were displayed as single-parameter DNA histograms. Ploidy was determined relative to the analysis results of *B. napus* and *B. nigra* which served as external standards measured before analysis of the hybrid samples in every flow cytometry run. Main peak positions of standards and samples were compared, and samples showing a main peak between the peak positions of the diploid control *B. nigra* and the allotetraploid control *B. napus* were considered to be triploid (3*x*). Samples showing a main peak at twice the relative DNA content of the triploid ploidy level (6*x*) were considered to be hexaploid. In vitro colchicine-treated plants often showed two large peaks at both the predicted 3*x* and 6*x* ploidy levels, indicating a chimeric genotype. Samples with unusually large peaks at the G2 (duplication of DNA before cell division cell stage) peak position were considered to be putatively chimeric. To distinguish between euploid and chimeric plants, the proportion of particles at the G1 relative to the G2 peak in the allotetraploid parent *B. napus* 'MSL' was used as an external standard. Up to 27% of the particles measured across both peaks were present in the G2 peak of the control, and hence plants with two peaks where the G2 peak exceeded 27% of particles were considered to be chimeric.

### Pollen viability and cytogenetic analysis

Pollen viability was assessed by staining pollen of two to four flowers per plant on glass slides using 1% acetocarmine solution. Pollen grains were observed using a Leica DMRE light microscope. Plump, darkly stained pollen grains were assumed to be viable, while lightly stained and/or small and shrivelledshriveled pollen grains were assumed to be non-viable. A minimum of 300 pollen grains were counted per flower.

Chromosomes were counted in mitotic cells from root tissue following the protocol of Snowdon et al. (1997) but using DAPI (4,6-diamidino-2-phenylindole) to stain chromosomes. To assess chromosome pairing during meiosis, pollen mother cells at diakinesis were analyzed. Inflorescences harvested between 9 and 11 am were immersed in 3:1 ethanol:acetic acid solution and incubated in the dark at room temperature for 24 h (Mason et al. 2014). After incubation, the inflorescences were rinsed two times with 50% ethanol and then stored in 50% ethanol in small glass flasks at 4 °C. For chromosome spread preparation, anthers were dissected from flower buds and transferred to microscope slides. Anthers were cut using a razor blade and pollen mother

cells were released into 12.5 µl 1% acetic acid-carmine solution by squeezing. Pollen mother cells were spread by applying heat using a spirit flame followed by squashing between the cover glass and microscope slide. A minimum of 40 triploid and hexaploid pollen mother cells (PMCs) from 2 to 5 buds per cross combination were characterized for the number of univalent, bivalent, trivalents and higher multivalents at diakinesis, using a Leica DRME light microscope at × 1000 magnification.

Sixty-four triploid PMCs from three cross combinations were analyzed using BAC-FISH and GISH chromosome staining to differentiate the three subgenomes according to the protocol presented in Mason et al. (2010). Hybridization with the two labelled probes hybridizing to the B- and C- genome chromosomes was separated into two subsequent steps to avoid interference of the *B. napus* blocker DNA with the BAC-derived probe annealing to C-genome chromosomes.

## Seed production and cross pollination

Seeds of first generation *B. napus* × *B. nigra* hybrids were produced by self-pollination using micro-perforated plastic bags to prevent pollen contamination and by manual self-pollination of flowers before opening. Intercrossing of allohexaploid *B. napus* × *B. nigra* hybrids and backcrossing of allohexaploid hybrids to *B. napus* cultivars were conducted by manual emasculation of flowers followed by pollination with the selected crossing partner. Backcrossing of putative hexaploid hybrids of *B. napus* and *B. nigra* was performed using the *B. napus* accessions 'Argyle' (Canola Breeders Western Australia), 'Boomer', 'Darmor' (Australian Grains Genebank ATC90553), 'Express', 'Ningyou7' and 'Yudal' (provided by Anne-Marie Chèvre, INRA Rennes, France).

## *Leptosphaeria maculans* resistance testing

Cotyledon reactions of the parental cultivars of the *B. napus* × *B. nigra* hybrids against 11 individual field isolates were analyzed in separate assays. These field isolates were collected at different locations throughout Germany, and were previously race-typed on two differential sets consisting of ten oilseed rape genotypes (Supplementary Table 1). The differentials with known major resistance genes (*Rlm1–4, 7, 9; LepR1–3*) were provided by R. Delourme (INRA Rennes, France) and H. Borhan (AAFC, Saskatoon, Canada). Per isolate four seedlings were inoculated 7 days after germination by injuring cotyledon tissue with a sterile needle and applying 10 µl pycnidiospore suspension ($10^7$ spores/ml) at each side of the cotyledon midrib. The symptoms were scored 14 days post inoculation according to the IMASCORE rating (Van De Wouw et al. 2009; Volke 1999) where 1 represents a resistant reaction, 2 and 3 represent intermediate resistance and 4, 5 and 6 show increasing levels of susceptibility. Seedlings of the *B. napus* cultivar 'Lirabon' were used as a susceptible control. Seeds of the male-sterile *B. napus* cultivar 'MSL' (the female parent of most of the hybrids) germinated poorly, such that only two seedlings could be used per isolate for resistance testing.

Stem reaction was tested on two putative hexaploid hybrids per cross combination, except for the cross combination 'Ningyou7' × 'IX7', where only one hybrid was available to be tested. Hybrids were tested together with the susceptible control 'Westar' and the parental cultivars 'MSL', 'Ningyou7', 'Boomer', 'IX7', 'IX13' and 'Junius'. Each hybrid was cloned by cultivation of cuttings to produce six technical replicates for each hybrid. Isolate 1.4.1.15 was chosen to test stem resistance due to its virulence against all major R genes mentioned above. Controls and parental cultivars were sown in advance to the propagation of the hybrids from cuttings to produce plants of equal developmental stage. Inoculation was carried out at the stem base, where an incision was made with a sterile needle and an agar plug colonized with fungal mycelium was placed on the incision site. Length and girdling of the diseased tissue were measured 21, 35 and 49 days after inoculation. At the last date, stems of the plants were cut through the infection site to measure the depth of the diseased tissue. The volume of the diseased tissue (VDT) (Kutcher et al. 1993) was calculated as a measure to differentiate resistance/susceptibility of the different hybrids and cultivars. VDT was calculated according the following formula: $VDT = (1 - HR^2) \times G/9 \times L$; $HR = 1 - P/9$; where HR = the radius of healthy tissue in the cross section of the stem, G = girdling of the stem, $L$ = lesion length and $P$ = profundity of necrosis: the original rating scale was adapted to ranks between 0 and 9 (Supplementary Table 2).

## Statistical analysis

Statistical analysis was done in R Studio v3.4.2 (R Studio Team 2018). Plots for visualisation of ovules

produced per 100 pollinations, karyotype distribution after in-vitro colchicine treatment, pollen viability per karyotype across populations and backcross success rates were produced using the "ggplot2" package in R (Wickham 2016). To select the appropriate statistical test, data distribution was tested visually with QQplots and by performing Shapiro-Wilk tests. Homogeneity of variance was tested using Bartlett's test. When normal distribution and homogeneity of variance could be assumed, data was analysed using analysis of variance (ANOVA) with Tukey's Honest Significant Differences as a post-hoc test. In the cases where neither normality nor homogeneity of variance could be assumed, pairwise Wilcoxon tests were conducted followed by a correction for multiple testing based on false discovery rate (FDR). The distribution of karyotypes per cross combination after in vitro colchicine treatment was analysed using Fisher's exact test for count data in R version 3.4.4 (R Core Team 2018). Analysis of backcross seed production success between different hexaploid *B. napus* × *B. nigra* hybrids was done using the six success rates of the combinations with different *B. napus* cultivars as replicate to infer general backcrossing success rates per allohexaploid hybrid cross.

## 3. Results

### Pollination, embryo rescue and in vitro colchicine treatment

Pollination of the male sterile *B. napus* cultivar 'MSL' with three different *B. nigra* accessions produced 216 developed ovules, of which 35% further developed into a total of 76 triploid hybrid plants (Table 1). The *B. nigra* accession used for pollination significantly influenced the pollination success rate in these cross combinations (ANOVA; $p = 0.00515$). Pollination with the *B. nigra* accessions 'IX7' and 'IX13' produced the most developed embryos per pollination, while pollination using the accession 'Junius' produced significantly fewer developed embryos (Table 1).

Germination of embryos occurred as early as 6 days after dissection of the ovules. Embryos developed normally into seedlings with differentiated roots and cotyledons (Supplementary Fig. 1). However, 34% of the developing seedlings showed initially disrupted development of true leaves and shoots, observable in the form of excessive growth of cotyledons and hypocotyl-like tissue, or by hyper-hydration of plant tissue (Supplementary Fig. 2). Only 15% of the malformed plants failed to recover, while the other plants eventually produced normal shoots after repeated cutting and subculture of plants on MS medium (Murashige and Skoog 1962) without supplementation of plant hormones.

Additionally, to the main experiment using male-sterile *B. napus* as female parent, crosses were made using pollen fertile *B. napus* cultivars 'Boomer', 'Ningyou7', 'Monty_28DH' and 'Express'. Only the combinations 'Boomer' × 'IX7' and 'Ningyou7' × 'IX7' successfully produced ovules that further developed into plants. Ovules of the other combinations died after dissection from the ovaries (Table 1).

Ninety putative triploid hybrids developed after embryo rescue, 79 of which developed until flowering. The triploid karyotype was confirmed in 60 hybrids by flow cytometry or by chromosome counting. Mature plants showed more similarity to *B. napus* than to *B. nigra*, but differed from *B. napus* in the number of leaf lobes and in leaf serration (Fig. 1). Initial high trichome density of true hybrids (Supplementary Fig. 1) reduced over time and was not as pronounced in mature plants. Triploids did not show strong phenotypic differences to the allohexaploid hybrids derived from them. Hybrids that were not subjected to in vitro colchicine treatment were grown to maturity, and inflorescences were covered using micro-perforated plastic bags to ensure self-pollination. None of the putative triploid hybrids produced seeds by self-pollination, further confirming that all hybrids developed by embryo rescue were true triploid hybrids.

In vitro colchicine treatment of plants derived from five different cross combinations produced 27 putative allohexaploid hybrid plants: 29% of the treated plants remained triploid, 58% became chimeric and 13% became hexaploid (Table 1). The distribution of karyotypes between the different cross combinations was significantly different (Fisher's Exact Test $p = 0.000169$), indicating a significant influence of parental cultivars used in the cross combinations (Supplementary Fig. 2). Statistical testing of the distribution between cross combinations sharing the same female *B. napus* cultivar 'MSL' showed that the *B. nigra* accession used for pollination significantly influenced the colchicine treatment outcome (Fisher's Exact test, $p = 0.000161$). In vitro colchicine-treated plants

**Table 1** Number of pollinations performed between different *Brassica napus* and *B. nigra* accessions, embryo rescue and colchicine treatment of $F_1$ *Brassica napus* × *B. nigra* hybrids. "IX7" and "IX13" refer to Nordgen accessions NGB 21858.1 and NGB 23253.2 respectively. *n.a.*, not applicable; no hybrids were treated in the respective cross combinations

| *B. napus* × *B. nigra* cross combination | Bud pollinations | Developed ovules | Ovules/ pollination | Plants developed from ovules | Clones subjected to in vitro colchicine treatment[1] | In-vitro colchicine-treated plants | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | triploid | chimeric | hexaploid |
| 'MSL' × 'IX7' | 377 | 89 | 0.24 | 30 | 61 | 8 | 49 | 4 |
| 'MSL' × 'IX13' | 322 | 106 | 0.33 | 30 | 62 | 28 | 26 | 8 |
| 'MSL' × 'Junius' | 335 | 21 | 0.06 | 16 | 53 | 12 | 27 | 14 |
| 'Boomer' × 'IX7' | 9 | 33 | 3.67 | 13 | 31 | 13 | 17 | 1 |
| 'Boomer' × 'Junius' | 44 | 17 | 0.39 | 0 | n.a. * | n.a. | n.a. | n.a. |
| 'Ningyou7' × 'IX7' | 85 | 70 | 0.82 | 1 | 2 | 0 | 2 | 0 |
| 'Ningyou7' × 'Junius' | 102 | 10 | 0.10 | 0 | n.a. | n.a. | n.a. | n.a. |
| 'Monty' × 'IX7' | 79 | 109 | 1.38 | 0 | n.a. | n.a. | n.a. | n.a. |
| 'Monty' × 'Junius' | 68 | 25 | 0.37 | 0 | n.a. | n.a. | n.a. | n.a. |
| 'Express' × 'IX7' | 35 | 2 | 0.06 | 0 | n.a. | n.a. | n.a. | n.a. |
| 'Express' × 'Junius' | 12 | 0 | 0.00 | 0 | n.a. | n.a. | n.a. | n.a. |
| Total | 1468 | 482 | Av: 0.67 | 90 | 209 | 61 | 121 | 27 |

[1] Clones were developed by cutting in vitro grown plants: 3 to 6 plantlets were developed from 10 hybrids in each cross combination

differed in flower size, with some producing small flowers equal to the size of *B. nigra* flowers, and others producing larger flowers usually with well-developed anthers and elevated pollen viability (Fig. 1).

## Meiotic stability in triploid and hexaploid hybrids of *B. napus* and *B. nigra*

Meiosis in both the triploid and the hexaploid hybrids was highly irregular in all hybrid cross combinations (Fig. 2). The average frequency of univalent chromosomes in the triploid hybrids ranged from 6.9 to 8.8 univalents (out of 27 chromosomes; Table 2). Univalent frequency was significantly different between both cross combinations sharing the same *B. napus* parent and between cross combinations sharing the same *B. nigra* parent, indicating that both *B. napus* and *B. nigra* genotype influenced meiotic chromosome pairing in the triploid hybrids. The average bivalent frequency between cross combinations ranged from 5.9 to 7.3, with significant differences between the cross combinations Boomer × IX7 (5.9) and MSL × IX7 (7.2) (pairwise Wilcoxon test and FDR correction $p < 0.05$) suggesting that genomic factors contributed by the *B. napus* genome caused variation in bivalent frequency in the triploid hybrids (Table 2). The frequency of trivalents per pollen mother cell ranged from 1.1 to 1.8, and multivalents with more than three chromosomes occurred at a frequency between 0.1 and 0.4 per PMC (Table 2).

Analysis of allohexaploid PMCs revealed an average univalent frequency between 1.4 and 2, and a bivalent frequency between 20.5 and 21.7. Trivalents occurred at an average frequency between 1.5 and 2.3 per PMC. Multivalents with more than three chromosomes occurred at a frequency between 0.7 and 1.1 per PMC. The only significant difference in meiotic pairing between genotypes was found in the frequency of trivalents, where the cross combination MSL × IX7 produced significantly more trivalents per pollen mother cell than the three other cross combinations (pairwise Wilcoxon test with FDR correction $p < 0.05$; Table 2).

BAC-FISH/GISH staining was conducted on triploid pollen mother cells in metaphase I from three cross combinations: 42 cells were analysed from the cross 'MSL' × 'IX7', 24 cells from the cross 'Ningyou' × 'IX7', and 14 cells from the cross 'Boomer' × 'IX7'. In a total of 80 stained pollen mother cells, 30 cells (38%) showed allosyndetic pairing involving B-genome chromosomes in the triploid hybrids. B-A allosyndetic pairing was observed in 23% of the analysedanalyzed cells, and 16% of the cells showed B-C allosyndetic pairing. Autosyndesis in triploid hybrids between B-genome chromosomes was observed in 5% of PMCs.

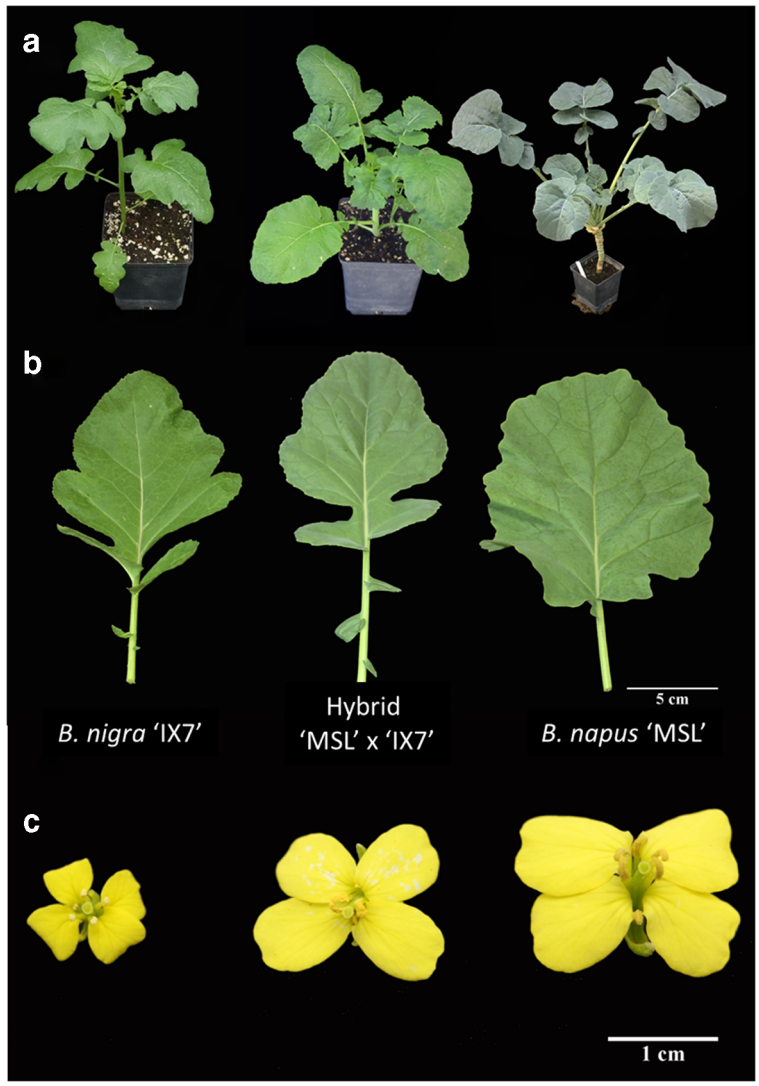



**Fig. 1** Plant phenotype, leaf morphology and flower morphology of *B. napus* "MSL", *B. nigra* NBG 21858.1 ("IX7") and their interspecific hybrid. **a** *B. nigra* 'IX7' (left), triploid hybrid of *B. nigra* 'IX7' × *B. napus* 'MSL' (middle) and *B. napus* 'MSL' (right). **b** Leaf morphology of the triploid hybrid and its parents. **c** Flower morphology of *B. nigra* 'IX7' (left), the triploid hybrid between *B. nigra* 'IX7' and 'MSL' (middle) and the fertile hexaploid hybrid from the same cross combination (right)

In the allohexaploid hybrids, 92 pollen mother cells in diakinesis (54 PMCs) or metaphase I (38 PMCs) were analysed after chromosome staining. Plants from three cross combinations were used: 53 cells from plants of the cross combination 'MSL' × 'IX13', 49 cells from plants of 'MSL' × 'IX7' and two cells from 'MSL' × 'Junius' were analysed. Similar frequencies of allosyndetic pairings were observed in diakinesis (8 observations in 54 PMCs) and in metaphase I (6 observations in 38 PMCs). In 15.2% of PMCs B-genome chromosomes were paired with A (8 cells; 8.7%) or C (6 cells; 6.5%) genome chromosomes. A maximum of two such allosyndetic chromosome pairs were found per PMC. Most homeologous chromosome pairing took the form of allosyndetic multivalent (more than two chromosomes) chromosome pairs (60%), while the remainder (40%) of allosyndesis was found in bivalent chromosome pairs. Between one and four univalent chromosomes were observed in 13% of the analysed pollen mother cells.

*Leptosphaeria maculans* resistance testing of parental cultivars and interspecific hybrids

The two parental *B. napus* cultivars 'Ningyou7' and 'Boomer' showed susceptibility on cotyledons towards all 11 field isolates tested, except for isolates 1.4.1.15 and 2.4.1.29 against which the cultivar 'Boomer' showed some tolerance (Supplementary

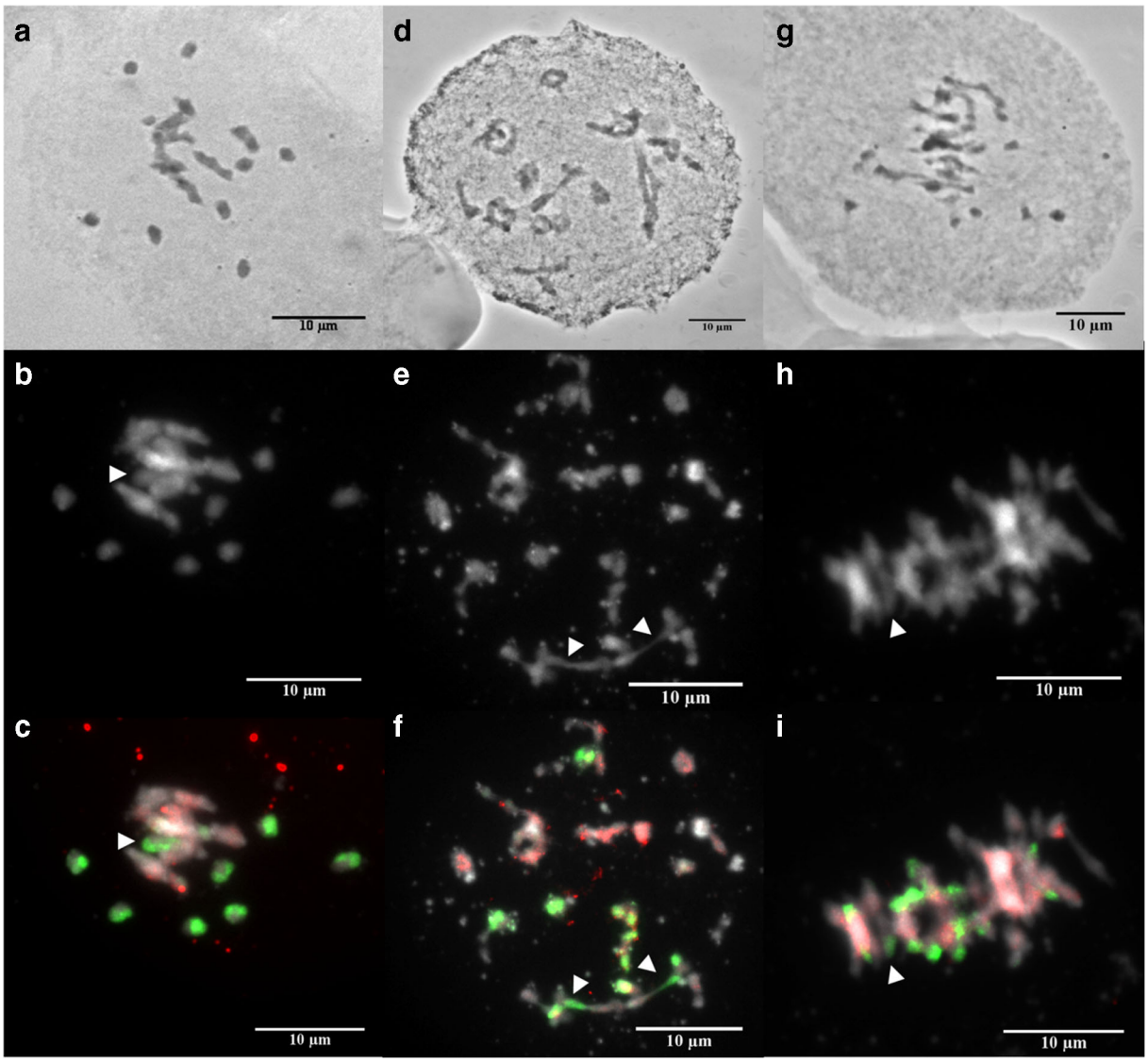



**Fig. 2** Cytogenetic analysis of triploid and hexaploid *Brassica napus* × *B. nigra* hybrids. **a** Triploid meiosis in metaphase showing 9 univalents and 9 bivalents. **b**, **c** Triploid meiosis showing B-C allosyndetic (►) pairing. Green signals (GISH) are derived from genomic *B. nigra* DNA probes and highlight B-genome chromosomes, while red signals (FISH) are derived from repetitive *B. oleracea* DNA probes highlighting C-genome chromosomes. **d** Hexaploid meiosis in diakinesis showing 18 bivalents, 2 trivalents and 3 quadrivalents. **e**, **f** Diakinesis cells showing allosyndetic (►) quadrivalent pairing of two B-genome and two A-genome chromosomes. **g** Hexaploid meiosis in metaphase showing 9 univalents, 18 bivalents and 3 trivalents. **h**, **i** Metaphase cell showing allosyndetic B-A bivalent formation (►)

Fig. 3). Testing of the *B. napus* cultivar 'MSL' could only be done on two seedlings per isolate due to poor germination of seeds, precluding conclusive analysis. However, all 'MSL' seedlings inoculated showed strong susceptible reactions to all 11 isolates. The three *B. nigra* accessions tested all showed strong resistance against all tested *L. maculans* field isolates.

Stem resistance testing of the parental cultivars showed contrary results to the cotyledon testing (Fig. 3). While the *B. napus* cultivar 'MSL' showed strong susceptibility in the cotyledon inoculation test, 'MSL' showed resistance against the isolate (1.4.1.15) used in the stem inoculation tests, and 'Boomer' showed similar resistance to 'MSL'. All *B. nigra* accessions showed similar resistance, as

**Table 2** Meiotic pairing of triploid and allohexaploid *Brassica napus* ("MSL", "Boomer" and "Ningyou7") × *B. nigra* ("IX7", "IX13" and "Junius") hybrids in diakinesis. "IX7" and "IX13" refer to Nordgen accessions NGB 21858.1 and NGB 23253.2 respectively. Indices [a-c] show significant differences in univalent, bivalent, trivalent and higher multivalent frequencies between cross combinations after pairwise Wilcoxon tests and FDR correction for multiple testing

| | Genotype | No. of cells analysed | Average univalent frequency (range) | Average bivalent frequency (range) | Average trivalent frequency (range) | Frequency of higher multivalents (range) |
|---|---|---|---|---|---|---|
| Triploid | 'MSL' × 'IX7' | 43 | 8.8$^{bc}$ (6–12) | 7.2$^{a}$ (5–10) | 1.1 (0–2) | 0.1 (0–1) |
| | 'MSL' × 'IX13' | 40 | 7.4$^{a}$ (2–9) | 6.5$^{ab}$ (2–10) | 1.8 (0–4) | 0.3 (0–2) |
| | 'MSL' × 'Junius' | 40 | 6.9$^{a}$ (3–10) | 7.3$^{a}$ (4–12) | 1.4 (0–3) | 0.3 (0–2) |
| | 'Boomer' × 'IX7' | 40 | 8.3$^{ac}$ (4–14) | 5.9$^{b}$ (3–9) | 1.8 (0–4) | 0.3 (0–1) |
| | 'Ningyou7' × 'IX7' | 46 | 7.7$^{a}$ (5–10) | 6.6$^{ab}$ (4–10) | 1.6 (0–4) | 0.3 (0–1) |
| Hexaploid | 'MSL' × 'IX7' | 45 | 2 (0–8) | 21.5 (18–26) | 1.5$^{b}$ (0–4) | 0.9 (0–3) |
| | 'MSL' × 'IX13' | 40 | 1.4 (0–4) | 21.7 (17–25) | 2.2$^{a}$ (0–4) | 0.7 (0–3) |
| | 'MSL' × 'Junius' | 40 | 1.7 (0–5) | 20.5 (13–25) | 2.3$^{a}$ (0–4) | 1.1 (0–3) |
| | 'Boomer' × 'IX7' | 41 | 1.7 (0–8) | 21.6 (17–27) | 2$^{a}$ (0–5) | 0.8 (0–2) |
| | 'Ningyou7' × 'IX7' | n.a. | n.a. | n.a. | n.a. | n.a. |

did all interspecific hybrids tested. Only *B. napus* parent 'Ningyou7' showed susceptibility to isolate 1.4.1.15. However, the interspecific hybrid between 'Ningyou7' and 'IX7' showed strong stem resistance to isolate 1.4.1.15, demonstrating that resistance conferred by the *B. nigra* B genome is expressed in the interspecific hybrids.

## Pollen viability and seed fertility in self-, cross- and backcross pollination

In vitro colchicine-treated plants varied greatly in flower morphology, flower size, and pollen viability. Forty-three percent of the colchicine-treated plants failed to produce an inflorescence, either because they died before reaching maturity or due to developmental issues. The morphology of anthers and the flower size of the remaining plants were assessed repeatedly over a time frame of 2 months after the plants began to flower. Forty-nine percent of the plants produced flowers with normally developed anthers, but flower development was strongly influenced by environmental factors such as temperature in the greenhouse. Sterile flowers without normally developed anthers or senescence of flower buds were observed in 33% of the plants that previously showed normal flower development. Pollen viability was assessed in 29% of the colchicine-treated plants. Pollen viability ranged between 0 and 10% in the triploid plants, between 0 and 69% in the chimeric plants and between 0 and 88% in the hexaploid plants, with no statistical difference between groups (Kruskal-Wallis rank sum test, $p = 0.53$). However, fertile hexaploid plants often showed larger flowers with higher pollen viability than chimeric and triploid plants (Fig. 1).

Only seven plants (all chimeras) from the cross combinations 'MSL' × 'IX7' and 'Boomer' × 'IX7' produced a small number of seeds by self-pollination (1–23 seeds). No plants in the other cross combinations produced seeds using this method. Self-pollination by hand was not successful even in hybrids that previously produced seeds when inflorescences were covered with micro-perforated plastic bags. Backcrossing of allohexaploid hybrids from four cross combination was much more successful than self-pollination in obtaining seeds: in total, 1655 seeds have already been obtained. Hexaploid plants from the cross combinations 'Boomer' × 'IX7' and 'MSL' × 'IX13' showed the largest average seed set per backcross pollination (1.6 and 1.0 respectively), but did not differ significantly to each other. Hexaploid hybrids from the cross combination 'MSL' × 'IX7' produced on average 0.2 seeds per pollination by backcrossing, and a hexaploid plant from the cross combination 'MSL' × 'Junius' produced the least number of seeds per backcross pollination (0.03, Fig. 4). Hexaploid *B. napus* × *B. nigra* hybrids of different cross combinations were also cross-pollinated to produce heterozygous allohexaploid $F_1$ hybrids, producing 113 seeds. Pollination success rates ranged from 0.42 to 0.79 seeds per pollination.

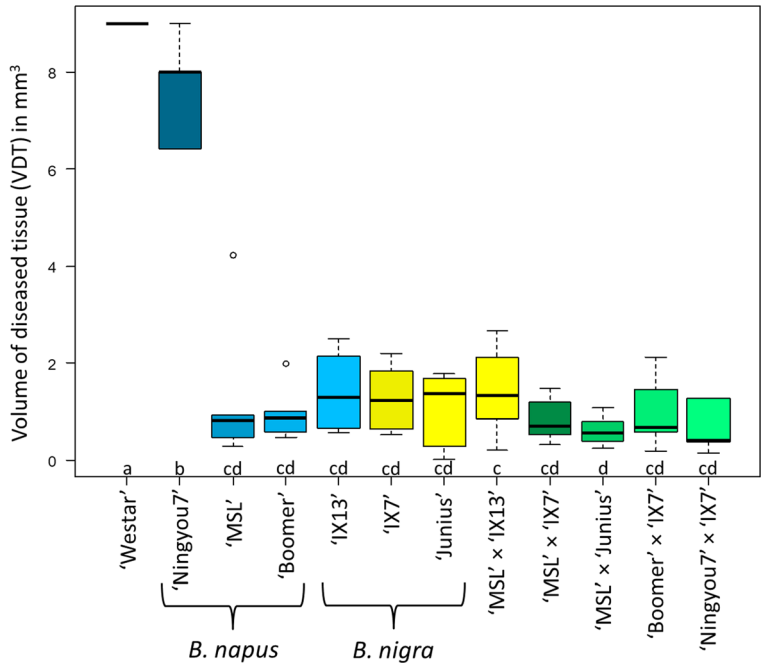



**Fig. 3** Adult plant resistance of a susceptible *Brassica napus* control ('Westar'), parental *B. napus* and *B. nigra* accessions and the hybrids between them against *L. maculans* isolate 1.4.1.15. Letters a–c indicate significant differences in the volume of diseased tissue in $mm^3$ (VDT) after inoculation (Wilcoxon pairwise rank sum test comparison and false discovery rate (FDR) correction for multiple testing, $p < 0.05$). "IX7" and "IX13" refer to Nordgen accessions NGB 21858.1 and NGB 23253.2 respectively

## 4. Discussion

The application of embryo rescue proved to be highly successful in obtaining true hybrids between *B. napus* and *B. nigra* in comparison to other studies. Depending on the cross combination, 2–9% of pollinations resulted in the development of a triploid hybrid plant. In one case, pollination of only nine flowers produced 13 true hybrids (cross combination 'Boomer' × 'IX7'). Altogether, 90 putative triploid hybrid plants were produced, 60 of which were confirmed to have a triploid karyotype; the remaining hybrids were not tested but their morphology and the fact that they failed to produce seeds by self-pollination suggests that they were also triploid. Busso et al. (1987) reported the successful production of six triploid *B. napus* × *B. nigra* hybrids, while Jahier et al. (1989), Ge et al. (2009) and Pradhan et al. (2010a) all report successful production of one true hybrid Numbers of pollinations needed to produce true hybrids are rarely reported with sufficient clarity to assess the difficulty of this cross combination. Pradhan et al. (2010a) report that one true hybrid was produced after 799 bud pollinations when *B. napus* was used as female parent. The frequent occurrence of matromorphic offspring in crosses between *B. napus* and *B. nigra* found by Pradhan et al. (2010a) was not observed in our experiment, neither in the crosses of male-sterile winter-type *B. napus* with *B. nigra* nor in the crosses of male-fertile spring-type *B. napus* with *B. nigra*. Not all of the cross combinations tested in our experiments produced triploid hybrids, which suggests a strong influence of parental genotype on hybridization success. Overall, the use of embryo rescue appeared to be the most important factor in improving hybridization success compared to other studies. Between 3 and 32% of the triploids we subjected to in vitro colchicine treatment became hexaploid, and the *B. nigra* genotype in the different cross combinations was found to significantly influence the outcome of the in vitro colchicine treatment. Struss et al. (1991) and Pradhan et al. (2010b) successfully produced one and two true hexaploid hybrids via colchicine application, the only hexaploid *B. napus* × *B. nigra* hybrids reported in the scientific literature until now.

Fertility of our allohexaploid hybrids was very low: only 55 seeds were produced by self-pollination of seven plants from two cross combinations ('MSL' × 'IX7' and 'Boomer' × 'IX7'). Seed set could have been influenced by the male sterility of the female *B. napus* parent of the hybrid 'MSL', or by self-incompatibility of the *B. nigra* parents. The male sterility of 'MSL' is temperature-sensitive, such that fertility and anther

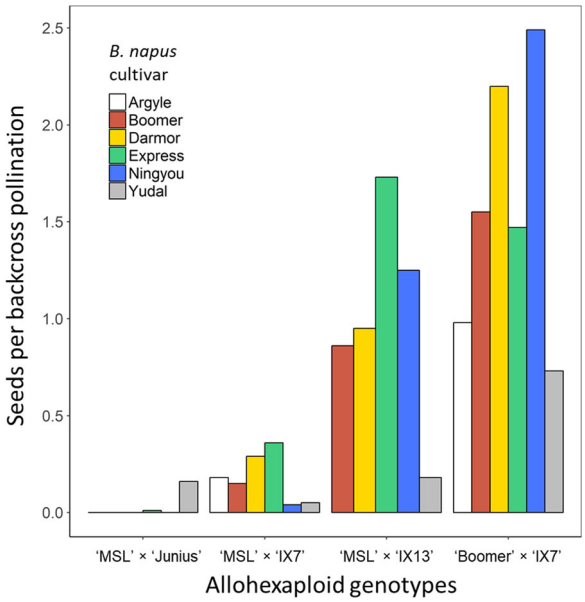



**Fig. 4** Seeds produced from different genotypes of allohexaploid hybrids (*Brassica napus* cultivars 'MSL' and 'Boomer' crossed with *B. nigra* accessions 'Junius', 'IX7' and 'IX13') after backcrossing to different *B. napus* cultivars ("Argyle", "Boomer", "Darmor", "Express", "Ningyou7" and "Yudal"). "IX7" and "IX13" refer to Nordgen accessions NGB 21858.1 and NGB 23253.2 respectively

development are restored at high temperatures (ca. 35 °C) (Stiewe et al. 2009), which were potentially reached in the greenhouse during the summer growing season. In support of this, a number of plants showed a mix of fertile and sterile flowers. The *B. nigra* accessions used also showed strong but not complete self-incompatibility. However, although seed set was improved when hexaploid hybrids of different cross combinations were cross pollinated, this improvement was not striking enough to suggest that self-incompatibility was the main factor influencing hybrid fertility, although it may still play a role. We suspect that the main cause of the low fertility of the allohexaploid hybrids is irregular meiosis, which matches observations of both low fertility and highly irregular meiosis in most other early generation allohexaploid *Brassica* hybrid types (Li et al. 2015; Zhou et al. 2016; Mason et al. 2014). Fertility and meiosis were also clearly linked in *B. rapa* by *B. carinata* allohexaploids: Gupta et al. (2016) found 100% bivalent formation in the most highly fertile lines in this cross combination, but only 10–85 seeds per plant for most lines with irregular meiosis. The allohexaploid *B. napus* × *B. nigra* hybrids of Struss et al. (1991) and Ge et al. (2009) did not produce any seeds by self-pollination. No information on self-pollinated seed set by the hexaploid hybrid produced by Pradhan et al. (2010b) is published, although this hybrid was able to produce seeds when crossed with other allohexaploid hybrids (Geng et al. 2013).

Meiosis in both the triploid and the hexaploid hybrids was found to be highly irregular. Significant differences in chromosome pairing configurations of the triploid plants appeared to be caused by genetic factors coming from both parents. This is the first time to our knowledge that genotypic variation from *B. nigra* has been reported to influence meiosis. In *B. napus*, allelic variation in *PrBn* (pairing homeologous *Brassica napus*), is known to influence the frequency of allosyndetic pairing in haploid *B. napus* accessions, as indicated by different frequencies of univalents during meiosis (Cifuentes et al. 2010). A similar mechanism could be operating in the different frequencies of univalents and bivalents in the triploid hybrids of the different cross combinations. Univalent frequencies in our study ranged from 6.9 to 8.8, which is comparable to the results of Jahier et al. (1989). Busso et al. (1987) reported frequencies of between 10.4 and 11.7 univalents per PMC in triploid ABC hybrids from different species cross combinations. We observed non-homologous chromosome pairing between B and A/C chromosomes in 38% of PMCs in our *B. napus* × *B. nigra* triploids (Fig. 2b, c): this is similar to the 45% of PMCs reported by Ge and Li (2007) who report at least one B-A/C allosyndetic bivalent in ABC triploids from the cross combination *B. napus* 'Zhongyou 821' × *B. nigra* 'Giebra'.

Hexaploid hybrids also demonstrated low meiotic stability, particularly by the high frequency of univalent and multivalent chromosomes present (Fig. 2d, g). The only significant difference in meiotic pairing between cross combinations was found in the frequency of trivalents per pollen mother cell, which ranged between 1.5 and 2.3. Overall, the cross combination did not influence meiotic paring in the allohexaploids. Previous studies by Busso (1985) and Struss et al. (1991) reported average chromosome pairing configurations in allohexaploid *B. napus* × *B. nigra* of 12.4I + 17.5II + 2.2III and 9.24I + 22.33II respectively. While we found much lower numbers of univalents per pollen mother cell and higher numbers of multivalents than both previous studies, the main reason for this may be that presented meiotic pairing frequencies were analyzed in cells in diakinesis while both Busso (1985) and Struss et al. (1991) relied on cells in metaphase. Cells in diakinesis

generally show less overlap of chromosomes, a big advantage over cells in metaphase in which chromosomes are often very densely packed at the equatorial plate (Fig. 2d, e); cells in diakinesis additionally better display multivalent chromosome pairing structures.

To our knowledge, no information on non-homologous chromosome pairing frequencies involving the B-genome chromosomes in hexaploid hybrids of *B. napus* and *B. nigra* has been published to date. The fact that B-genome chromosomes remain unpaired and even undergo non-homologous chromosome pairing (Fig. 2f, i) in 15.2% of the analyzed pollen mother cells highlights how severely meiosis is destabilized by the combination of the three subgenomes in the very first meiosis of this hybrid type. Whatever genetic factors that may be present in the *B. napus* genome that prevent A-C chromosome pairing do not affect chromosome pairing between the A/C and B-genome chromosomes. Previous studies suggested relatively stable meiotic pairing of B-genome chromosomes in triploid (BC.B) hybrids of *B. carinata* × *B. nigra* (Attia et al. 1987) as well as in trigenomic tetraploid (BBAC) hybrids of *B. carinata* × *B. juncea* (Mason et al. 2010). Cui et al. (2012) report low frequencies of B-genome allosyndesis in synthetic *B. juncea* and *B. carinata* hybrids, always occurring in the form of multivalents where a pair of B-genome chromosomes recombines with another homologous chromosome pair from the other genome. Allohexaploid hybrids from the cross *B. juncea* × *B. oleracea* as well as *B. carinata* × *B. rapa* were found to form normal B-genome bivalents in later generations, and the univalent frequency of B-genome chromosomes was always lowest when compared to univalents of the A and C genome (Zhou et al. 2016). However, the successful introgression of B-genome-derived traits such as silique shattering resistance (Dhaliwal et al. 2017) and *L. maculans* resistance (Barret et al. 1998; Rashid et al. 2018) to *B. napus* via interspecific hybridization with *B. juncea* and *B. carinata* supports the occurrence of non-homologous recombination in other hybrid types containing the A, B and C genomes, as also observed by Mason et al. (2010) in AABC, BBAC and CCAB hybrid types. B-genome chromosomes are also easily eliminated through backcrossing to *B. napus* (Meng et al. 1998), suggesting that if introgressions do occur, recovery of *B. napus*-type ($2n$ = AACC) plants carrying these introgressions should be feasible.

All *B. nigra* accessions were strongly resistant to blackleg infection at the cotyledon stage. Interestingly, while all *B. napus* cultivars used as parents were susceptible in cotyledon tests to isolate 1.4.1.15, the two cultivars 'Boomer' and 'MSL' were resistant in stem inoculation tests. A similar lack of correlation between cotyledon test results and stem infection test results was found by Kutcher et al. (1993) with respect to blackleg resistance in *B. napus*. The cultivar 'Ningyou7' was susceptible in both the cotyledon and stem inoculation tests. However, the triploid hybrid between 'Ningyou7' and 'IX7' was just as resistant as the *B. nigra* parent, showing clearly that the B-genome-derived resistance is active in the hybrid. Only one substantial attempt to produce *L. maculans*-resistant *B. napus* via introgression of *B. nigra* resistance has been documented in the literature. This attempt goes back to a single hybrid plant produced between *B. napus* 'Tandem' and *B. nigra* 'Junius' (Jahier et al. 1989). The triploid hybrid was backcrossed to *B. napus*, and a series of B-genome addition lines was developed (Chèvre et al. 1996). Assessment of resistance in early generation hybrids suggested that multiple loci were contributing to the resistance phenotype (Zhu et al. 1993), but analysis of later generation hybrids only report a single major resistance gene, possibly, due to loss of other loci (Brun et al. 2001). As of today, no publication conclusively shows that *B. nigra*-derived resistance against *L. maculans* has been successfully transferred to the *B. napus* genome via non-homologous recombination.

Both resistances from *B. nigra* and *B. juncea* were overcome in field trials in France by especially virulent strains of *L. maculans* (Brun et al. 2001; Sprague et al. 2006). However, the putatively monogenic resistances derived from the B genome were shown to be overcome more slowly when combined with quantitative (field-based) resistance from *B. napus* (Brun et al. 2010; Delourme et al. 2014). Hence, the potential of our interspecific hybrids to combine quantitative resistance from the *B. napus* parents with novel resistance from *B. nigra* could have significant agronomic benefit if introgression lines can be recovered. Not much is known about the diversity of *L. maculans* resistance genes in *B. nigra*, as resistance from only a single cultivar ('Junius') has been utilized prior to our study. Our material represents a promising start for further research to enhance the pool of blackleg resistance genes available for *B. napus* crop improvement. A new genotyping platform enabling the identification of B-genome alleles in interspecific *Brassica* hybrids is now available, and advances in sequencing technology have further closed the price gap between SNP-based

genotyping platforms and genotyping-by-sequencing approaches (Schiessl et al. 2018). These new technologies will allow fast identification of true genomic introgressions, pinpointing the exact location of non-homologous recombination breakpoints and allowing identification of resistance genes against *L. maculans* in B-genome interspecific hybrids.

**Acknowledgements** Advice on FISH/GISH chromosome staining and embryo rescue by Ghader Mirzhagaderi, Olivier Coriton and Anne-Marie Chèvre is gratefully acknowledged, as is the assistance and support in plant cultivation and cross pollination by Ingrid Schneider-Hüther and Petra Degen. Furthermore, we would like to thank Volker Wissemann for providing the infrastructure for flow cytometry.

**Author contributions** RG provided the experimental design advised by ASM, produced the hybrids, collected and analysed the meiotic and phenotypic data and wrote the manuscript. DA performed *L. maculans* resistance tests of parental cultivars by cotyledon inoculation and adult plant resistance tests of hybrids and parental cultivars, with input into experimental design, analysis and supervision from BK. ASM supervised RG and assisted in interpretation and analysis of results. All authors contributed to critical revision of the manuscript.

**Funding information** This work was supported by Deutsche Forschungsgemeinschaft (DFG) Emmy Noether grant MA6473/1-1.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal studies** This article does not contain any studies with human or animal subjects performed by any of the authors.

## References

Attia T, Busso C, Röbbelen G (1987) Digenomic triploids for an assessment of chromosome relationships in the cultivated diploid *Brassica* species. Genome 29:326–330. https://doi.org/10.1139/g87-053

Barret P, Guérif J, Reynoird JP, Delourme R, Eber F, Renard M, Chèvre AM (1998) Selection of stable *Brassica napus-Brassica juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*). 2. A "to and fro" strategy to localise and characterise interspecific introgressions on the *B. napus* genome. Theor Appl Genet 96:1097–1103. https://doi.org/10.1007/s001220050844

Brun H, Ruer D, Levivier S, Somda I, Renard M, Chèvre AM (2001) Presence in *Leptosphaeria maculans* populations of isolates virulent on resistance introgressed into *Brassica napus* from the *B. nigra* B genome. Plant Pathol 50:69–74. https://doi.org/10.1046/j.1365-3059.2001.00535.x

Brun H, Chèvre A-M, Fitt BD et al (2010) Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. New Phytol 185: 285–299. https://doi.org/10.1111/j.1469-8137.2009.03049.x

Busso C (1985) Analyse der Verwandschaftsbeziehnungen innerhalb der Gattung *Brassica* anhand der Chromosomenpaarung in verschiedenen Bastardkombinationen der drei Grundgenome. Dissertation, Georg-August University Göttingen

Busso C, Attia T, Röbbelen G (1987) Trigenomic combinations for the analysis of meiotic control in the cultivated *Brassica* species. Genome 29:331–333. https://doi.org/10.1139/g87-054

Chèvre AM, Eber F, This P et al (1996) Characterization of *Brassica nigra* chromosomes and of blackleg resistance in *B. napus-B. nigra* addition lines. Plant Breed 115:113–118. https://doi.org/10.1111/j.1439-0523.1996.tb00884.x

Chèvre AM, Brun H, Eber F, Letanneur JC, Vallee P, Ermel M, Glais I, Li H, Sivasithamparam K, Barbetti MJ (2008) Stabilization of resistance to *Leptosphaeria maculans* in *Brassica napus - B. juncea* recombinant lines and its introgression into spring-type *Brassica napus*. Plant Dis 92:1208–1214. https://doi.org/10.1094/pdis-92-8-1208

Cifuentes M, Eber F, Lucas M-O, Lode M, Chèvre AM, Jenczewski E (2010) Repeated polyploidy drove different levels of crossover suppression between homoeologous chromosomes in *Brassica napus* allohaploids. Plant Cell Online 22:2265–2276. https://doi.org/10.1105/tpc.109.072991

Cui C, Ge X, Gautam M, Kang L, Li Z (2012) Cytoplasmic and genomic effects on meiotic pairing in *Brassica* hybrids and allotetraploids from pair crosses of three cultivated diploids. Genetics 191:725–738. https://doi.org/10.1534/genetics.112.140780

Delourme R, Horvais R, Barret P, Renard M, Foisset N, Champagne G, Cheung WY, Landry BS (1998) Characterization of the radish introgression carrying the *Rfo* restorer gene for the *Ogu*-INRA cytoplasmic male sterility in rapeseed (*Brassica napus* L.). Acta Hortic 459:135–138. https://doi.org/10.17660/ActaHortic.1998.459.14

Delourme R, Chèvre AM, Brun H, Rouxel T, Balesdent MH, Dias JS, Salisbury P, Renard M, Rimmer SR (2006) Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). Eur J Plant Pathol 114:41–52. https://doi.org/10.1007/s10658-005-2108-9

Delourme R, Bousset L, Ermel M, Duffé P, Besnard AL, Marquer B, Fudal I, Linglin J, Chadœuf J, Brun H (2014) Quantitative resistance affects the speed of frequency increase but not the diversity of the virulence alleles overcoming a major resistance gene to *Leptosphaeria maculans* in oilseed rape. Infect Genet Evol 27:490–499. https://doi.org/10.1016/j.meegid.2013.12.019

Dhaliwal I, Mason AS, Banga S et al (2017) Cytogenetic and molecular characterization of B-genome introgression lines of *Brassica napus* L. G3 7:77–86. https://doi.org/10.1534/g3.116.036442

Gaebelein R, Mason AS (2018) Allohexaploids in the genus *Brassica*. CRC Crit Rev Plant Sci 1–16. https://doi.org/10.1080/07352689.2018.1517143

Ge XH, Li ZY (2007) Intra- and intergenomic homology of B-genome chromosomes in trigenomic combinations of the cultivated *Brassica* species revealed by GISH analysis. Chromosom Res 15:849–861. https://doi.org/10.1007/s10577-007-1168-4

Ge XH, Wang J, Li ZY (2009) Different genome-specific chromosome stabilities in synthetic *Brassica* allohexaploids revealed by wide crosses with *Orychophragmus*. Ann Bot 104: 19–31. https://doi.org/10.1128/JB.06078-11

Geng XX, Chen S, Astarini IA, Yan GY, Tian E, Meng J, Li ZY, Ge XH, Nelson MN, Mason AS, Pradhan A, Zhou WJ, Cowling WA (2013) Doubled haploids of novel trigenomic *Brassica* derived from various interspecific crosses. Plant Cell, Tissue and Organ Culture (PCTOC) 113(3):501–511

Gupta M, Atri C, Agarwal N, Banga SS (2016) Development and molecular-genetic characterization of a stable *Brassica* allohexaploid. Theor Appl Genet 129:2085–2100. https://doi.org/10.1007/s00122-016-2759-2

Hajjar R, Hodgkin T (2007) The use of wild relatives in crop improvement: a survey of developments over the last 20 years. Euphytica 156:1–13. https://doi.org/10.1007/s10681-007-9363-0

Howard HW (1942) The effect of polyploidy and hybridity on seed size in crosses between *Brassica chinensis*, *B. carinata*, amphidiploid *B. chinensis-carinata* and auto-tetraploid *B. chinensis*. J Genet 43:105–119

Huang YJ, Mitrousia GK, Sidique SNM, Qi A, Fitt BDL (2018) Combining R gene and quantitative resistance increases effectiveness of cultivar resistance against *Leptosphaeria maculans* in *Brassica napus* in different environments. PLoS One 13:1–22. https://doi.org/10.1371/journal.pone.0197752

Jahier J (1992) Techniques of plant cytogenetics. Science Publishers, New Hampshire

Jahier J, Chevre AM, Tanguy AM, Eber F (1989) Extraction of disomic addition lines of *Brassica napus* - *Brassica nigra*. Genome 32:408–413

Kuswinanti T, Koopmann B, Hoppe H-H (1999) Virulence pattern of aggressive isolates of *Leptosphaeria maculans* on an extended set of *Brassica* differentials. J Plant Dis Protect 106(1):12–20

Kutcher HR, van den berg CGJ, Rimmer SR (1993) Variation in pathogenicity of *Leptosphaeria maculans* on *Brassica* spp. based on cotyledon and stem reactions. Can J Plant Pathol 15: 253–258. https://doi.org/10.1080/07060669309501920

Li J, Rao L, Meng Q, Ghani MA, Chen L (2015) Production of *Brassica* tri-genomic vegetable germplasm by hybridisation between tuber mustard (*Brassica juncea*) and red cabbage (*B. oleracea*). Euphytica 204(2):323–333

Mason AS, Batley J (2015) Creating new interspecific hybrid and polyploid crops. Trends Biotechnol 33:436–441. https://doi.org/10.1016/j.tibtech.2015.06.004

Mason AS, Huteau V, Eber F, Coriton O, Yan G, Nelson MN, Cowling WA, Chèvre AM (2010) Genome structure affects the rate of autosyndesis and allosyndesis in AABC, BBAC and CCAB *Brassica* interspecific hybrids. Chromosom Res 18:655–666. https://doi.org/10.1007/s10577-010-9140-0

Mason AS, Yan G, Cowling WA, Nelson MN (2012) A new method for producing allohexaploid *Brassica* through unreduced gametes. Euphytica 186:277–287. https://doi.org/10.1007/s10681-011-0537-4

Mason AS, Nelson MN, Takahira J, Cowling WA, Alves GM, Chaudhuri A, Chen N, Ragu ME, Dalton-Morgan J, Coriton O, Huteau V, Eber F, Chèvre AM, Batley J (2014) The fate of chromosomes and alleles in an allohexaploid *Brassica* population. Genetics 197:273–283. https://doi.org/10.1534/genetics.113.159574

Meng J, Shi S, Gan L, Li Z, Qu X (1998) The production of yellow-seeded *Brassica napus* (AACC) through crossing interspecific hybrids of *B. campestris* (AA) and *B. carinata* (BBCC) with *B. napus*. Euphytica 103:329–333

Morinaga T (1934) Interspecific hybridization in *Brassica* VI. The cytology of F1 hybrids of *B. juncea* and *B. nigra*. Cytologia (Tokyo) 6:62–67. https://doi.org/10.1508/cytologia.6.62

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Pereira-Lorenzo S, Costa R, Anagnostakis S, et al (2016) Interspecific hybridization of chestnut. In: Mason AS (ed) Polyploidy and hybridization for crop improvement, pp 377-407. CRC Press, Boca Raton

Pradhan A, Plummer JA, Nelson MN, Cowling WA, Yan G (2010a) Trigenomic hybrids from interspecific crosses between *Brassica napus* and *B. nigra*. Crop Pasture Sci 61: 464–474. https://doi.org/10.1071/CP09276

Pradhan A, Plummer JA, Nelson MN, Cowling WA, Yan G (2010b) Successful induction of trigenomic hexaploid *Brassica* from a triploid hybrid of *B. napus* L. and *B. nigra* (L.) Koch. Euphytica 176:87–98. https://doi.org/10.1007/s10681-010-0218-8

Qiu W, Feechan A, Dry I (2015) Current understanding of grapevine defense mechanisms against the biotrophic fungus (*Erysiphe necator*), the causal agent of powdery mildew disease. Hortic Res 2:1–9. https://doi.org/10.1038/hortres.2015.20

R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

R Studio Team (2018) RStudio: Integrated development environment for R. Boston, MA. Available from http://www.rstudio.org/

Rashid MH, Zou Z, Fernando WGD (2018) Molecular and phenotypic identification of B-genome introgression linked to *Leptosphaeria maculans* resistance gene *Rlm6* in *Brassica napus* × *B. juncea* interspecific hybrids. Euphytica 214:1–11. https://doi.org/10.1007/s10681-018-2287-z

Rouxel T, Balesdent MH (2017) Life, death and rebirth of avirulence effectors in a fungal pathogen of *Brassica* crops, *Leptosphaeria maculans*. New Phytol 214:526–532. https://doi.org/10.1111/nph.14411

Schiessl SV, Katche E, Ihien E, Chawla HS, Mason AS (2018) The role of genomic structural variation in the genetic improvement of polyploid crops. Crop J 7:127–140. https://doi.org/10.1016/j.cj.2018.07.006

Snowdon RJ, Köhler W, Friedt W, Köhler A (1997) Genomic in situ hybridization in *Brassica* amphidiploids and interspecific hybrids. Theor Appl Genet 95:1320–1324. https://doi.org/10.1007/s001220050699

Soltis PS, Marchant DB, Van de Peer Y, Soltis DE (2015) Polyploidy and genome evolution in plants. Curr Opin

Genet Dev 35:119–125. https://doi.org/10.1016/j.gde.2015.11.003

Sprague SJ, Balesdent MH, Brun H, Hayden HL, Marcroft SJ, Pinochet X, Rouxel T, Howlett BJ (2006) Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. Eur J Plant Pathol 114:33–40. https://doi.org/10.1007/s10658-005-3683-5

Stiewe G, Pleines S, Coque M, Gielen J (2009) Neues Hybridsystem für *Brassica napus*. Patent application US20100222605A1

Struss D, Bellin U, Röbbelen G (1991) Development of B-genome chromosome addition lines of *B. napus* using different interspecific *Brassica* hybrids. Plant Breed 106:209–214. https://doi.org/10.1111/j.1439-0523.1991.tb00503.x

U N (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. Jap J Bot 7:389–453

USDA (2018) World Supply and Use of Oilseeds and Oilseeds Products. Oil Crop Yearbook. https://www.ers.usda.gov/data-products/oil-crops-yearbook/oil-crops-yearbook. Accessed 10 Jannuary 2019

Van De Wouw AP, Marcroft SJ, Barbetti MJ et al (2009) Dual control of avirulence in *Leptosphaeria maculans* towards a *Brassica napus* cultivar with "sylvestris-derived" resistance suggests involvement of two resistance genes. Plant Pathol 58:305–313. https://doi.org/10.1111/j.1365-3059.2008.01982.x

Volke B (1999) *Leptosphaeria maculans*, der Erreger der Wurzelhals- und Stengelfäule an Raps: Verbreitung verschiedener Pathogenitätsgruppen in Europa, Quantifizierung des Befalls und Schadwirkung im Freiland. PhD thesis, Georg-August-Universität Göttingen [German]

Wickham H (2016) ggplot2: elegant graphics for data analysis. Springer-Verlag, New York

Winter M, Koopmann B (2016) Race spectra of *Leptosphaeria maculans*, the causal agent of blackleg disease of oilseed rape, in different geographic regions in northern Germany. Eur J Plant Pathol 145(3):629–641. https://doi.org/10.1007/s10658-016-0932-8

Zhou J, Tan C, Cui C, Ge X, Li Z (2016) Distinct subgenome stabilities in synthesized *Brassica* allohexaploids. Theor Appl Genet 129:1257–1271. https://doi.org/10.1007/s00122-016-2701-7

Zhu JS, Struss D, Röbbelen G (1993) Studies on resistance to *Phoma lingam* in *Brassica napus-Brassica nigra* addition lines. Plant Breed 111:192–197. https://doi.org/10.1111/j.1439-0523.1993.tb00629.x

# Occurrence of phytoalexins and phytoanticipins in *Brassica napus* and its correlation to quantitative resistance against *Leptosphaeria maculans* infection

Dima Alnajar[1], Mohammad Alhussein[2], Petr Karlovskey[2] and Birger Koopmann[1]

[1]Department of Crop Sciences, Division of Plant Pathology and Crop Protection, Georg-August University Göttingen, Göttingen, Germany
[2]Department of Crop Sciences, Division of Molecular Phytopathology and Mycotoxin Research, Georg-August University Göttingen, Göttingen, Germany

## Abstract

Oilseed rape is an important cruciferous crop grown mainly for its oil-rich seeds. It is a main host of the pathogen *L. maculans*, the causal agent of blackleg disease that can cause significant yield losses. Knowing the mechanisms of resistance of oilseed rape to *L. maculans* helps to find new crop protection strategies. Little research has explored the occurrence of plant defence chemicals in *B. napus* involved in quantitative resistance to blackleg. In this study, we investigated changes in the accumulation of phytoalexin and phytoanticipin blends upon infection with *L. maculans* in two *B. napus* genotypes, susceptible cultivar Lorenz and the resistant double haploid line DH378. Quantitative resistance of both genotypes was evaluated under greenhouse conditions. Development of lesions on stems of 40 plants was monitored at 7, 14 and 21 dpi. AUDPC and the profundity of the lesions at stem base on 21 dpi confirmed that DH378 is significantly more resistant than Lorenz. Plant defence chemicals were extracted with an acetone-water extraction method at similar timepoints as sampling for resistance phenotyping. Subsequently, the accumulation of caulilexin C, spirobrassinin, rutalexin, cyclobrassinin and brassilexin was compared between both genotypes using HPLC combined with high-resolution Q-TOF mass spectrometry. By comparing mock-inoculated with infected plants, caulilexin C was identified to be a phytoanticipin regardless of infection, while rutalexin, spirobrassinin, cyclobrassinin, and brassilexin were induced *de novo* upon infection, thus acting as phytoalexins. At 7dpi, all phytoalexins analysed revealed markedly higher concentrations in the resistant as in the susceptible genotype. These differences decreased at 14 and 21 dpi which might indicate that the early plant response to infection at 7dpi is correlated to smaller symptoms at stem level. Spirobrassinin, a phytoalexin that cannot be detoxified by *L. maculans*, showed the highest concentration among the investigated phytoalexins at all time points, while brassilexin was the lowest. The role of these metabolites in quantitative resistance needs to be further investigated by genetic methods such as the knock-down of genes in near isogenic lines.

# 1. Introduction

Oilseed rape (*B. napus*) is one of the most economically important species in the family *Brassicaceae* (Fitt et al. 2006). The pathogen *L. maculans* is a hemibiotrophic ascomycete. Its host range is strictly confined to *Brassica* crops. Infecting *B. napus,* the pathogen induces canker at the stem base and in severe cases causes lodging, which contributes to significant yield losses (Rouxel and Balesdent, 2005). Plant responses to *L. maculans* infection may range from a complete lack of pathogen growth mediated by qualitative resistance genes to gradual levels of partial quantitative disease resistance (QDR) reaching susceptibility (French et al. 2016).

QDR is known to be controlled by a number of genetic regions in the genome (QTLs) while qualitative resistance is operated by a major resistance (*R*) gene (Poland et al. 2009). Several studies showed that there is an overlap between the components of both types of resistance. In particular, it was shown that defeated major *R* genes or weak forms of *R* genes may add effects to quantitative resistance (Li et al. 1999; Fukuoka et al. 2015). There is also evidence that genes regulate plant growth and development contribute towards defining the level of QDR (Raman et al. 2016, 2018). This makes it more complex when defining QDR mechanisms. Yet, it is essential to study the correlation between different biological processes to understand the network of pathways that lead to improved plant health.

QDR is non-race specific and of partial influence. These characteristics are also assigned to, on the one hand, the passive plant defences, namely barriers such as the cuticle, the cell wall and the constitutively produced antimicrobials (phytoanticipins). On the other hand, these features can be seen in the basal defence mechanisms which are activated when a conserved microbe-associated molecular pattern (MAMP) is conceived. This can lead to pattern-triggered immunity (PTI) (Peyraud et al. 2017). PTI can affect several physiological responses such as $Ca^{+2}$ burst, ROS burst, regulation of transcriptional factors, transduction of phytohormonal defence signals such as salicylic acid, jasmonic acid and ethylene, and the induction of plant chemical warfare as phytoalexins (Poland et al. 2009; Bigeard et al. 2015). Interrogation of QDR mechanisms is a wide field. Hence, this work focused on the correlation between selected plant defence secondary metabolites and QDR in oilseed rape.

Plant secondary metabolites are fundamental phytochemicals for plant defence and fitness, but are not essential for plant growth, development, and reproduction (Hartmann, 2008). Phytoalexins and phytoanticipins are defence antimicrobial secondary metabolites. Phytoalexins are induced de novo by a biotic or abiotic stressor after activation of a gene or more that mediate a phytoalexin pathway. On the contrary, phytoanticipins or their precursors

exist constitutively in plants and provide them with protective readiness to cope with stresses even before being exposed to a stressor (Agrios, 2005; Pedras and Yaya, 2010).

Phytoalexin blends and concentrations change in plants after infection continuously so infection development analysis can show different mixtures at each point in time. A study of the rice- *Magnaporthe oryzae* interaction showed that the accumulation of the diterpene phytoalexins (i.e. momilactones A and B, and phytocassanes A through E) started on the second day post inoculation (dpi) and increased by up to 500-1000 fold at four dpi (Hasegawa et al. 2010). Additionally, phytoalexins occurrence differs among plant tissues and is concentrated mostly at the site of infection. For instance, phytoalexins induced by *P. brassicae* were observed in the roots of *B. napus* but not in leaves (Pedras et al. 2008c).

Numerous experiments studying plant resistance mechanisms have recognized that phytoalexins and phytoanticipins are of the main components of resistance mechanisms (e.g. He and Dixon, 2000; Jeandet et al. 2013). To prove causation relationship between resistance and phytoalexin induction, it was suggested to take two steps. Firstly, to confirm a correlation between the accumulation of the phytoalexins and the resistance level of the plant, followed by genetic modulation methods to examine whether there is a causative connection to this resistance (Poland et al. 2009). Loss-of-function and gain-of-function approaches, that target RNA, DNA or proteins were used extensively to confirm a causative relation (Ibraheem et al. 2010; Hipskind and Paiva, 2000).

Phytopathogens have developed mechanism to circumvent the toxic effect of phytoalexins during coevolution with their hosts. For instance, *L. maculans* showed ability to detoxifiy a wide range of phytoalexins, namely brassinin, cyclobrassinin, brassilexin, brassicanal A, sinalexin, and brassicanate A. Nonetheless, it fails to detoxify spirobrassinin and rapalexin A (Su et al, 2020; Pedras and Taylor, 1993; Pedras and Khan, 1996; Pedras and Suchy, 2005; Ahuja et al. 2012; Pedras and Sarma-Mamillapalle, 2012; Pedras and Abdoli, 2017). Su et al. (2020) illustrated that pathogens differ in their detoxification efficacies. Bioassays showed evidence that *L. maculans* detoxify brassinin two-fold compared to *V. longisporum* after incubation for 72 h. In contrast, *V. longisporum* was more efficient in detoxifying brassilexin compared to *L. maculans* six hours after incubation (Su et al, 2020). Detoxification of phytoalexins can be mediated by oxidase, reductase or hydrolase fungal enzymes (Pedras et al. 2011). For example, *L. maculans* might detoxify brassinin through brassinin hydrolase (BHLm), or via brassinin oxidase (BOLm) (Pedras et al. 2008a; Pedras et al. 2009). Yet, some plants can inhibit detoxifying enzymes by producing other chemicals that are known as paldoxins (**p**hyto**al**exin **d**et**oxi**fication **in**hibitors). An example is the function of cyclobrassinin as a paldoxin against brassinin hydrolase BHLm (Pedras et al. 2009).

Several biochemical studies investigated the direct interaction of *L. maculans* with specific phytoalexins in Petri dishes. However, such studies do not consider the interconnected complex of biological processes that occur in a plant during the infection. Indeed, a pathogen might be sensitive to specific phytoalexins at an early stage of infection, while its sensitivity differs in a later stage (Heitefuss, 1982). Also, the localisation of phytoalexins at the infection site or their systematic spread impacts the outcome of the interaction (Heitefuss, 1982). Other studies compared the ability of phytoalexin production in a resistant and a susceptible cultivar to blackleg by inducing phytoalexin production using $CuCl_2$ as a stressor rather than *L. maculans* (Rouxel et al, 1991).

More studies that investigate the induction of phytoalexin due to *L. maculans* infection are necessary. Indeed, Dahiya and Rimmer, 1988 investigated the occurrence of two phytoalexins, cyclobrassinin and methoxybrassinin after inoculating detached leaves and segments of stem cuts with *L. maculans*. However, over the last thirty years many more phytoalexins were commercially synthesised and are available as standards to be accurately defined in plant tissues. Therefore, in this study we aimed to investigate the induction of some of the most prevalent phytoalexins in *B. napus* that are documented in the literature (Pedras et al. 2008c). We compared these defence chemicals in *B. napus- L. maculans* dynamic pathosystem over time in a susceptible and a resistant genotype using a HPLC-Q-TOF that allowed for higher sensitivity and the detection of small amounts of phytoalexins in comparison with the NMR technique used by Dahiya and Rimmer, 1988.

## 2. Materials and methods

### 2.1 Plant material and fungal strain

Two *B. napus* genotypes were used in this experiment: *B. napus* cv. Lorenz that shows no QDR in the field and harbors the major *R* gene *Rlm9*, and the double haploid line DH387 produced from crossing cv. Lorenz and cv. King 10. King 10 is known to show QDR in the field and it has the *RLm4* major *R* gene. Both genotypes were provided by the breeding company Norddeutsche Pflanzenzucht (NPZ), Holtsee, Germany.

To meet the goal of this experiment by defining the phytoalexin induction in correlation with resistance at stem level, we chose an isolate whose interactions with plants at the cotyledon was not hypersensitive in its reaction. Instead, it overcame the resistance presented by *Rlm9* in Lorenz, *Rlm4* in DH387 or other potential *R* genes and grew further to the stem. For this, we tested a set of ten isolates that were provided from the Division of Plant Pathology and Crop Protection, Department of Crop Sciences, University of Göttingen, Germany. Ten µl of spore suspension ($10^7$ spores/ml) were used to inoculate the cotyledons of seven-day old

seedlings of Lorenz and DH387. Three replicates per genotype and isolate were inoculated. The inoculum was placed on a point injury of the lobes of each cotyledon. Monitoring was conducted 14 days post inoculation (dpi) according to the IMASCORE scale, where class one was given for plants that showed hypersensitive reactions and six for plants whose cotyledon tissues collapsed, producing more than ten pycnidia. Isolates that were assessed with scores five or six were well suited for this experiment. Based on the results of the cotyledon test, *L. maculans* isolate 1.4.1.15 was selected. Isolate 1.4.1.15 was virulent on cotyledons of both Lorenz and DH387 with classes 5.5 and 5, respectively. Isolate 1.4.1.15 was isolated from phoma lesions in a field experiment in Peine in a previous project in autumn 2013. It was characterized as an isolate not harboring the functional avirulence genes *AvrLm1, AvrLm2, AvrLm4, AvrLm7, AvrLm9, AvrLepR2,* and *AvrLepR3.*

### 2.2 Phenotyping in greenhouse and sampling procedure

Seeds were sown in trays containing a soil mixture of garden soil (Fruhstorfer Erde, Type P, pH 5.5- 5.6; Hawita-Gruppe, Vechta, Germany): steamed compost: sand at a ratio of 3:3:1. Seven days after germination plants were transferred to pots (13 cm * 13 cm) and filled with a the same soil mixture. Inoculation was conducted three weeks after transplanting. To prepare the inoculum, the spore suspension of the isolate 1.4.1.15 was plated ten days before inoculation on an oatmeal agar medium (2% oatmeal, 1.5% agar and 200 ppm streptomycin) and incubated at room temperature in darkness. Five-millimeter mycelia plugs were cut by a cork borer at the border of the colonies. To inoculate the stem, plants were superficially injured at the stem base and a mycelial agar plug or an agar plug was applied as a control. Plants were placed in a foilar tunnel for 72 hours to allow saturated humid conditions to develop for an optimal *L. maculans* infection. Plants were grown at 20°C and 16/8 day/light system using HPS 400W lamps (Hortilux Schreder, Monster, The Netherlands). Phenotyping for quantitative resistance and sampling for phytoalexin analysis was conducted at 7dpi, 14 dpi and 21 dpi.

For phenotyping, a percentage of the stem girdled by the lesion (G) and the lesion´s length (L) was scored based on a 9-class scale (Table 1). For stem girdling, plants of class one showed no infection, and stems of class nine > 88% girdled circumference. For the lesion length classification, class one means no infection while class nine indicated that lesion length was equal to or exceeded 32 mm. Disease severity (S) was presented as a sum of score values (S = G + L) according to (Newman, 1984) at 7, 14 and 21 dpi. Area Under Disease Progress Curve (AUDPC) was calculated at 21 dpi as followed (Madden et al., 2017):

$$\mathrm{AUDPC} = \sum_{j=1}^{n_i-1}\left(\frac{y_j + y_{j+1}}{2}\right)\left(t_{j+1} - t_j\right)$$

Values are calculated until $t_i$ = 21 dpi. Additionally, lesion profundity was also monitored at 21 dpi. Forty plants per time point were phenotyped for QDR.

To sample for chemical analysis, stems were cut 5 mm away from the border of the lesion and 5 mm from the point of injury for the control plants (Figure 1). Eight plants were pooled to form one replicate. Five replicates were analysed per treatment.


**Figure 1.** Sampling procedure

**Table 1.** Evaluation scale of *L. maculans* symptoms on oilseed rape stem bases modified from Kutcher et al. (1993)

| Class | Length (mm) | Girdling (%) |
|---|---|---|
| 0 | no infection | no infection |
| 1 | 1-4 | > 0-11% |
| 2 | 5-8 | 12- 22% |
| 3 | 9-12 | 23- 33% |
| 4 | 13-16 | 34- 44% |
| 5 | 17- 20 | 45- 55% |
| 6 | 21-24 | 56-66% |
| 7 | 25- 28 | 67- 77% |
| 8 | 29- 32 | 78-88% |
| 9 | >32 | >88% |

## 2.3 Secondary metabolites extraction

Samples were placed directly in liquid nitrogen to stop any further plant reactions after chopping the stems. Samples were lyophilized for 24 hours and subsequently ground at room

temperature. Two-hundred milligrams of ground powder was suspended in three ml of acetone-water (9:1, v/v) and shaken for 45 minutes. Afterwards, samples were centrifuged for ten minutes at 8000 x *g*, and 350 µl of the supernatant was transferred to fresh tubes. Acetone was evaporated using a speed vacuum centrifuge at 30 °C. Samples were then stored at – 80 °C until analysis.

Shortly before analysing the phytoalexin content, samples were dissolved in methanol: water (1:1, v/v). After incubation at room temperature for 30 min, samples were centrifuged at 16000 x *g* for ten minutes to remove any remaining tissue debris.

### 2.4 Analysis of secondary metabolites using HPLC - MS/MS

Phytoalexin analysis was conducted in the Division of Molecular Phytopathology and Mycotoxin Research, Department of Crop Sciences, University of Göttingen, Germany. HPLC-MS/MS analysis was carried out using a 1290 Infinity II high performance liquid chromatography (HPLC) system (Agilent Technologies, Waldbronn, Germany) combined with an Agilent 6460 triple quadrupole detector (Agilent Technologies, Waldbronn, Germany).

To separate the extracts, a Zorbax Eclipse Plus C18 column, of 50 x 2.1 mm diameter and 1.8 µm particle size (Agilent Technologies, Waldbronn, Germany) was used. Column temperature was adjusted to 40°C and a sample volume of 5 µl was injected. Solvent A consisted of water with 0.1% formic acid [v/v], and solvent B was methanol with 0.1% formic acid [v/v]. The gradient was set as follows: 0 to 0.2 min, 25% B; 0.2 to 8 min, 25% to 50% B; 8 to 10 min, 50% to 98% B; 10 to 13 minutes, 98% B; 13 to 13.5 minutes, 98% to 25% B; 13.5 to 16 minutes, 25% B. The column was operated at a 0.4 ml/minute flow rate.

An electrospray ionization (ESI) source was used to ionize the eluent under 4000 V capillary voltage, 60 psi Nebulizer pressure and a 350°C nitrogen inert gas with a flow rate of 13 l/minute. The multiple reactions monitoring (MRM) mode was used to selectively quantify phytoalexins in the samples. Values for tested samples were defined based on a calibration curve consisting of 14 concentrations from 0.49 to 4000 ng/mL. LOD and LOQ were established based on the standard deviation of the blank.

Standards for the secondary metabolites caulilexin C, rutalexin, spirobrassinin, cyclobrassinin and brassilexin were used. Brassilexin, cyclobrassinin and rutalexin were synthesized in the Division of Molecular Phytopathology and Mycotoxin Research, University of Göttingen, Germany. Spirobrassinin was provided by Dr. Mariana Budovska, Department of Organic Chemistry, Pavol Jozef Šafárik University, Slovakia. Caulilexin C was purchased from ChemFaces Biochemicals Co. Ltd., Wuhan, China.

### 2.5 Statistical analysis

Statistical analysis was performed using XLSTAT, version 2021.3.1. Data analysis of disease severity, AUDPC, and phytoalexins content was carried out using the Kruskal-Wallis test. For post hoc analysis, the Steel-Dwass-Critchlow-Fligner procedure was conducted at a significance level of 0.05.

## 3. Results

### 3.1 Phenotyping for quantitative resistance

One out of the ten isolates was chosen for this research. Ten isolates were tested on cotyledons of Lorenz and DH387. Based on the results shown in Table 2 we concluded that the isolate 1.4.1.15 was the most suitable for this experiment. That is because the isolate 1.4.1.15 was virulent on cotyledons of both genotypes with a mean of 5.5 and 5 calculated from 16 values (4 plants × 4 Lobes) on Lorenz and DH387, respectively. Thus, this isolate was able to overcome any gene-for-gene interaction at cotyledon level in the tested genotypes. As a result, the resistance that was afterwards evaluated at stem level is related to quantitative resistance.

**Tabel 2.** Cotyledon tests of a set of *L. maculans* isolates on two genotypes of *B. napus*: Lorenz and DH387. Values means of IMASCORE classes evaluated from three replications.

| Isolate code | Lorenz | DH387 |
|---|---|---|
| 2.5.1.02 | 5 | 5 |
| 2.7.1.06 | 6 | 3 |
| 1.1.1.55 | 6 | 4 |
| 1.4.1.15 | 5,5 | 5 |
| 2.2.1.28 | 4 | 3,5 |
| 2.4.1.29 | 5 | 5 |
| 2.2.1.29 | 5 | 4 |
| 1.1.1.57 | 6 | 4 |
| 1.4.1.65 | 6 | 4 |
| 1.1.1.66 | 6 | 4 |

This study aimed not only to describe the occurrence of a set of phytolaexins in *B. napus* but also to compare the differences between susceptible and resistant cultivars in phytoalexin induction upon infection. Therefore, we evaluated quantitative resistance of both genotypes before conducting chemical analysis. Phoma lesions were monitored at plant´s stem bases at 7, 14 and 21 dpi. Mock-inoculated plants of both genotypes showed no symptoms at any point in time, while infected plants presented symptoms starting from 7 dpi. DH378 had significantly less disease severity (S) and thus smaller lesions in comparison to Lorenz at all time points

(Figure 2A, 2B). Also, AUDPC values calculated at 21 dpi demonstrated that DH378 had significantly higher QDR than Lorenz (Figure 2C).

Additionally, stems were crosscut at the base of the stem at 21 dpi to evaluate the profundity of the lesion (Figure 2E). This characteristic is more pronounced in the field when assessing adult resistance against *L. maculans* due to sever yield losses in *B. napus* caused by the fungus blocking the vascular system in the stem preventing the transportation of nutrients. In line with AUDPC values, results confirmed that the lesion in Lorenz expanded significantly deeper in the stem than the lesion on DH378 (Figure 2D).

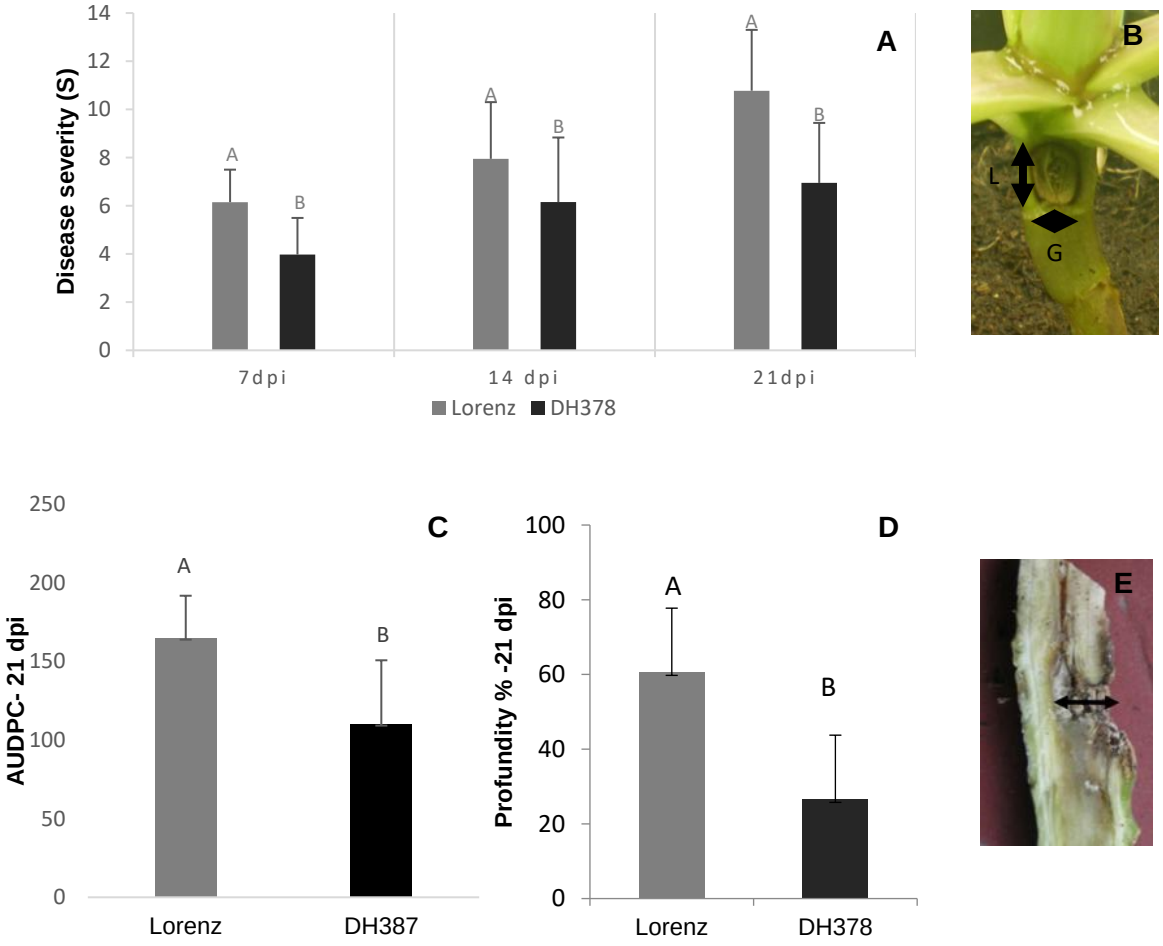



**Figure 2.** Phenotyping of *B. napus* cv. Lorenz and DH line 378 for quantitative resistance against *L. maculans*. Phoma lesions at the stem base of 40 plants were phenotypically evaluated. (A) Disease severity was estimated as a sum of lesion girdling scores (G) and lesion length (L). G and L were scored based on 1 to 9 classes. (B) Lesion length and girdling. (C) Area Under Disease Progress Curve (AUDPC). (D) Profundity of phoma lesion on *B. napus* stem base. (E) Lesion profundity. Letters highlight significant differences according to the Kruskal-Wallis test followed by post hoc comparisons with the Steel-Dwass-Critchlow-Fligner procedure ($P < 0.05$).

### 3.2 Phytoalexins and phytoanticipins in *B. napus-L. maculans* interaction

This study demonstrated that caulilexin C exists in concentrations of between 6.2 µg/kg and 19.6 µg/kg constitutively in intact oilseed rape stem bases regardless the infection with *L. maculans* or wounding the plants. There were no significant differences in caulilexin C content between the two genotypes before infection. It was concluded that caulilexin C is not a phytoalexin in *B. napus* stem base but rather a phytoanticipin (Figure 3A). In contrast, our analysis verified that rutalexin, spirobrassinin, cyclobrassinin and brassilexin are phytoalexins in the *B. naups-L. maculans* pathosystem. Their values were under the limit of detection (<LOD) in both intact plants (data not shown) as well as the injured mock-inoculated plants but were actively produced in the inoculated plants (Figure 4, 5, 6 and 7).

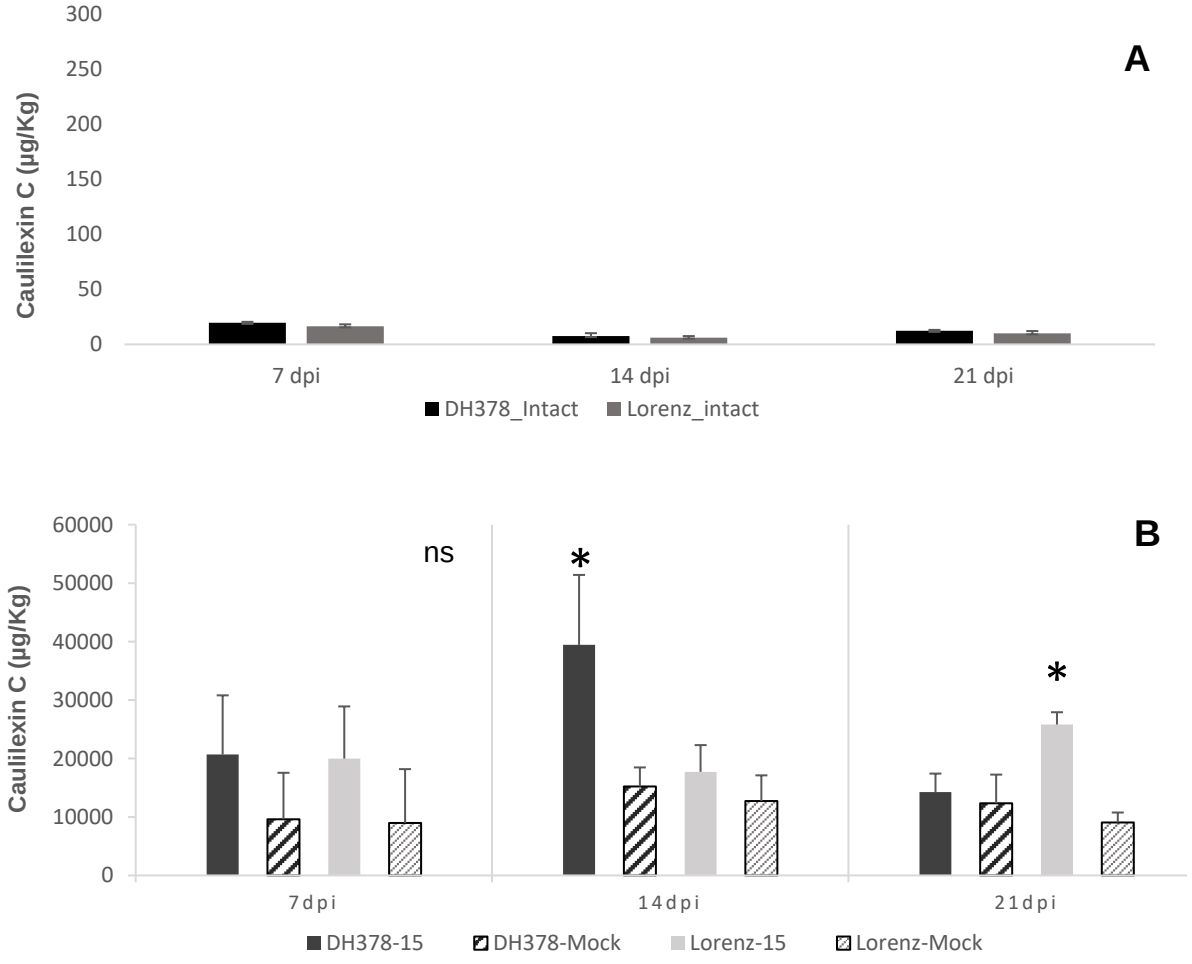



**Figure 3.** Caulilexin C content in samples taken from *B. napus* stem bases. (A) Caulilexin C content in stem bases before infection with *L. maculans* and without mechanical injury. (B) Comparison of caulilexin C content in plants inoculated with a mycelial agar plug on an injured stem base and mock-inoculated plants. Statistical analysis was conducted using the Kruskal-Wallis Test followed by post hoc comparisons with the Steel-Dwass-Critchlow-Fligner procedure ($P < 0.05$), ns= not significant. Asterisks and ns reflect the significance between the treatments in each time point separately.

Injuring the plants by mock-inoculation increased the amount of caulilexin C in *B. napus* significantly from 19.6 µg/kg to more than 8000 µg/Kg (Figure 3A and 3B). However, inoculating *B. napus* stems with *L. maculans* doubled the amount of caulilexin C compared to the amount observed in the mock-inoculated plants at 7 dpi. Yet, there were no significant differences between the susceptible and resistant genotypes (Figure 3B). At 14 dpi, however, the upsurge of caulilexin C was significantly higher in the infected but resistant genotype DH 378 in comparison to the infected susceptible Lorenz genotype (Figure 3B). The concentration of caulilexin C decreased in the infected DH378 plants at 21 dpi to the same level registered in the mock-inoculated DH378 genotype, whereas the level of caulilexin C continued to increase in the infected Lorenz genotype, reaching a significantly higher level than that of both the infected-DH378 and the mock-inoculated Lorenz genotypes (Figure 3B). Interestingly, the phenotypical evaluation in Figure 2A observed that the development of phoma lesions slowed down on stem base of DH378 after 14 dpi (S=6,1 at 14 dpi and S=6,9 at 21 dpi). In comparison, the lesions on Lorenz continued to expand after 14 dpi (S=7.9 at 14 dpi and S=10.8 at 21 dpi). The high level of caulilexin C production in the infected DH378 at a relatively early stage of the infection compared to Lorenz might have contributed to the significant reduction of *L. maculans* symptoms by the resistant genotype.

The resistant genotype DH378 produced significantly more rutalexin than the susceptible Lorenz genotpye at 7 dpi. However, the amount of rutalexin decreased significantly at 14 and 21 dpi in both genotypes with no significant difference between them. This indicates that rutalexin might play a role in the interaction between *B. napus* and *L. maculans* but only at an early stage of the infection (Figure 4).

Among the phytoalexins investigated in this study, spirobrassinin was the highest in both DH378 and Lorenz at 7 dpi, 14 dpi and 21 dpi (Figure 8). At 7 dpi, the resistant genotype produced notably more spirobrassinin than the susceptible one although the differences were statistically not significant. The stem content of spirobrassinin was insignificantly reduced at 14 dpi then slightly increased at 21 dpi in both Lorenz and DH378 (Figure 5).

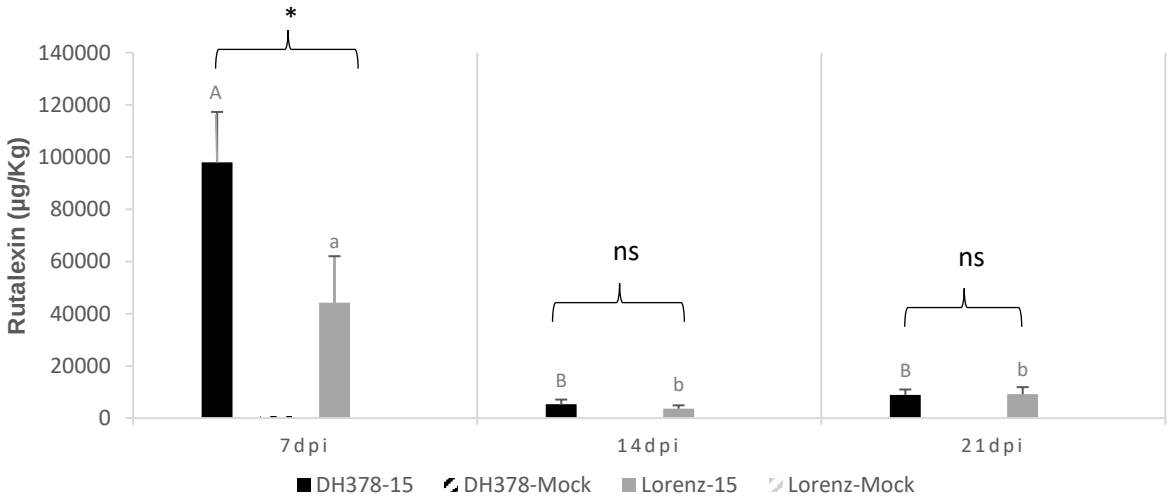



**Figure 4.** Rutalexin accumulation in *B. napus*-injured stems inoculated with an agar plug of *L. maculans* mycelium or mock-inoculated. All statistical analyses were conducted using the Kruskal-Wallis test followed by post hoc comparisons with the Steel-Dwass-Critchlow-Fligner procedure (P< 0.05); ns=not significant. Asterisks and ns reflect the significance between the treatments in each time point separately. Capital letters present the differences in rutalexin accumulation in DH378 over time, while small letters show these differences in accumulation in the infected Lorenz genotype. Values under the limit of detection were replaced with zeros.

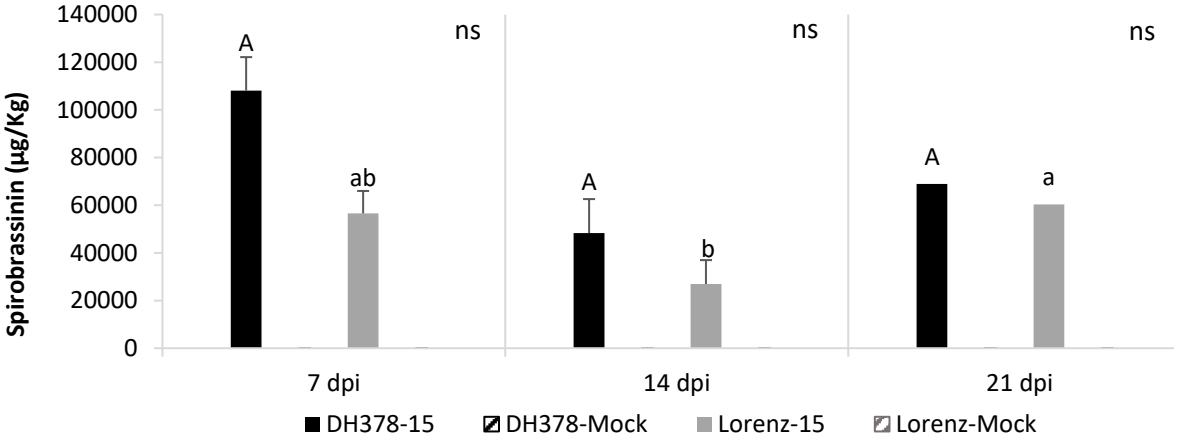



**Figure 5.** Spirobrassinin induction in oilseed rape stem bases in response to *L. maculans* infection or in mock-inoculated plants. All statistical analysis was conducted using the Kruskal-Wallis test followed by post hoc comparisons with the Steel-Dwass-Critchlow-Fligner procedure (P< 0.05); ns= not significant. Asterisks and ns reflect the statistical analysis between infected genotypes per time point. Capital letters present the differences in spirobrassinin accumulation in DH378 over time, while small letters show the differences in the infected Lorenz genotype. Values under the limit of detection were replaced with zeros.

Cyclobrassinin and brassilexin were accumulated in both genotypes as early as 7 dpi (Figure 6 and 7). Brassilexin showed the lowest concentration among the other phytoalexins in this study across all timepoints (Figure 8). Although with low concentration, brassilexin showed significant increases in DH378 in comparison to Lorenz at 7 dpi (Figure 7). Cyclobrassinin was the third dominant phytoalexin at 7 dpi and the second most dominant one at 14 dpi and 21 dpi (Figure 8). Cyclobrassinin concentration was significantly higher in the resistant genotype than the susceptible one at 7 and 14 dpi (Figure 6).

To sum up, this study described the occurrence of some phytoalexins and phytoanticipin in *B. napus*. The analysis clearly showed that the plants had reacted to the infection by inducing these defence chemicals as early as 7 dpi with the appearance of the symptoms on the stem. This study cannot confirm whether the accumulation started before the symptoms were visible. It was shown that the infection site had high concentrations of caulilexin C, rutalexin, spirobrassinin, cyclobrassinin and a low amount of brassilexin at 7 dpi. Caulilexin C increased significantly at 14 dpi in the resistant genotype. On the contrary, the significant accumulation of the other metabolites mainly peaked at 7 dpi (Figure 8).

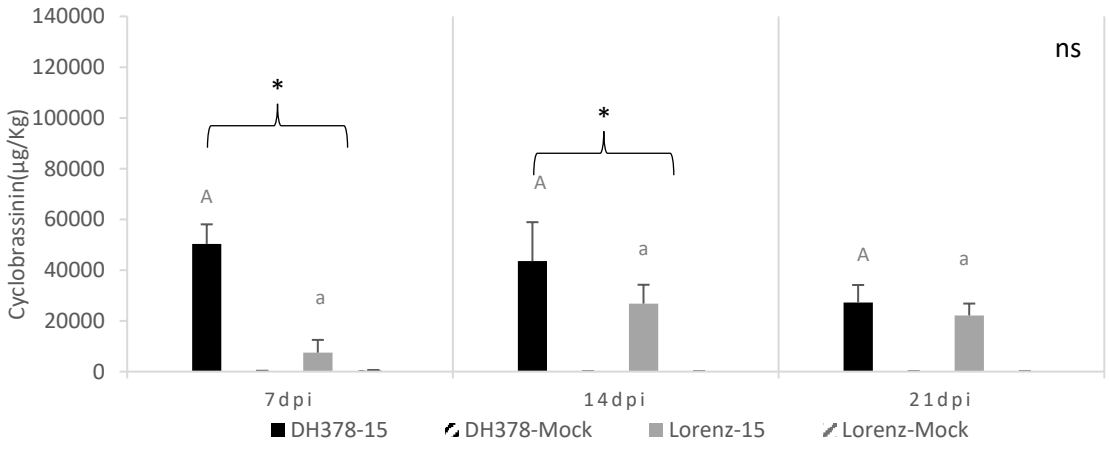



**Figure 6.** Cyclobrassinin induction in *B. napus -L. maculans* pathosystem. Plants were inoculated with a mycelial agar plug on an injured stem base. All statistical analysis was conducted using the Kruskal-Wallis Test followed by post hoc comparisons with the Steel-Dwass-Critchlow-Fligner procedure ($P< 0.05$); ns=not significant. Asterisks and ns reflect the statistical analysis between infected genotypes per time point. Capital letters present the differences in cyclobrassinin accumulation in DH378 over time, while small letters show the differences in infected Lorenz samples. Values under the limit of detection were replaced with zeros.

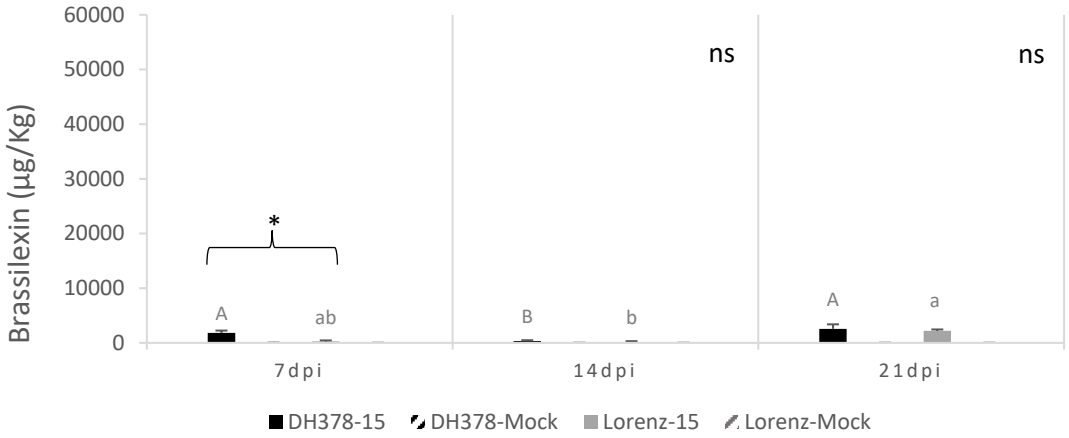



**Figure 7.** Brassilexin concentration in stem base samples of *B. napus* infected with *L. maculans*. All statistical analysis was conducted using the Kruskal-Wallis Test followed by post hoc comparisons with the Steel-Dwass-Critchlow-Fligner procedure ($P < 0.05$); ns=not significant. Asterisks and ns reflect the statistical analysis between infected genotypes per time point. Capital letters present the differences in brassilexin accumulation in DH378 over time, while small letters show the differences in infected Lorenz samples. Values under the limit of detection were replaced with zeros.

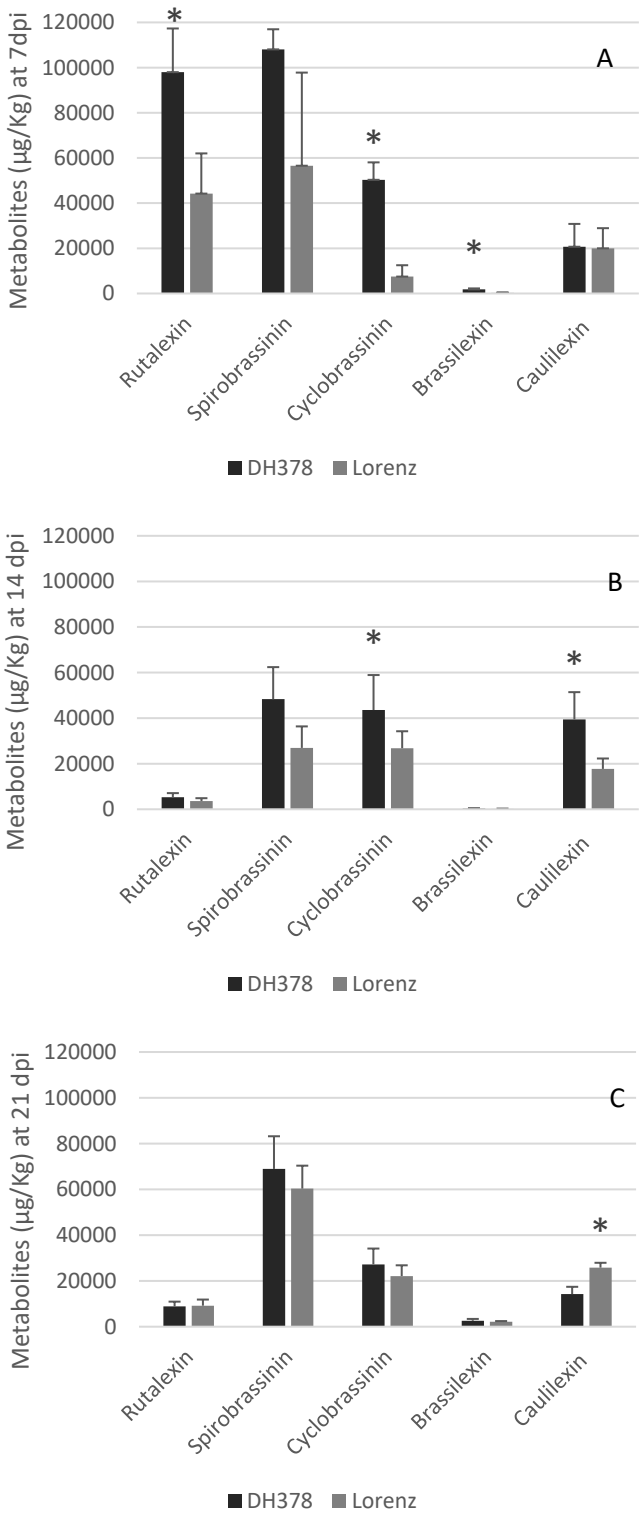



**Figure 8.** Occurrence of some phytoalexins and phytoanticipins in a resistant and susceptible *B. napus* genotypes infected with *L. maculans* at (A) 7 dpi, (B) 14 dpi, and (C) 21 dpi. All statistical analyses were conducted using the Kruskal-Wallis Test followed by post hoc comparisons with the Steel-Dwass-Critchlow-Fligner procedure ($P< 0.05$). Asterisks reflect significant differences between the susceptible *B. napus* cv. Lorenz and the resistant DH line 378.

## 4. Discussion

This study compared the changes in accumulation of the phytoalexins rutalexin, cyclobrassinin, spirobrassinin and brassilexin in *B. napus* stems infected with *L. maculans* during a series of timepoints in a resistant and susceptible genotype. It also documented the occurrence of the phytoanticipin caulilexin C in stems of *B. napus.* Caulilexin C has been shown to further accumulate in wounded plant stems and by *L. maculans* infection.

Caulilexin C was first detected in cauliflower after exposing slices of the florets to an abiotic elicitor (UV light), and was hence described as a phytoalexin (Pedras et al. 2006). Yet, a general categorisation of a plant chemical as a phytoanticipin or a phytoalexin can be misleading since one plant defence chemical can be found constitutively in some species but be induced upon infection in another. In line with Pedras et al. (2008c), who detected caulilexin C as phytoanticipin in *B. napus* roots*,* this study showed that caulilexin C is found in the stem of *B. napus* as phytoanticipin. In contrary, leaves of *B. napus* were free of caulilexin C before and after infection with *Plasmodiophora brassicae* (Pedras et al, 2008c).

Caulilexin C (or 1-mythoxy indolyl -3- acetonitrile) is a nitrile originated by hydrolysing the indolic glucosinolate 1-methoxyglucobrassicin by the myrosinase enzyme thioglucoside glucohydrolases to the intermediate indolic desulfo-glucosinolate 1-methoxydesulfo-glucobrassicin. The later would then be degraded to caulilexin C (Pedras and Hossain, 2011). The constitutively existing indolic glucosinolate and their desulfo-derivatives have no antibiotic effect *per se*, but they are rather the precursors for the antibiotic nitrile caulilexin C (Pedras and Hossain, 2011). The myrosinase enzyme is key to catalyse the hydrolysis reaction and they are stored in special idioblasts called myrosin cells. These are localized in ground tissues and in the phloem of *B. napus* (Andréasson et al. 2001). Injuring the plants may have been the mechanical releaser of the myrosinase from their compartments by rupturing myrosin cells. This might explain the increase of caulilexin C in the injured mock-inoculated plants unlike the phytoalexins studied here which remained undetectable despite injury.

*L. maculans* infects *B. napus* necrotrophically at stem level (Rouxel and Balesdent, 2005). This implies that infected cells are destroyed with their compartments. Consequently, more myrosinase could be released to hydrolyse the indole glucosinolate. This might explain that caulilexin C production was two-times higher in plants infected with *L. maculan*s as opposed to mock-inoculated plants at 7 dpi. In this case, plants were exposed to doubled destruction factors, biotically through the pathogen and abiotically through injury.

Radial growth antifungal bioassays in Petri dishes demonstrated that caulilexin C has an antifungal effect against *L. maculans, R. solani* and *S. sclerotiorum*. Pedras et al. 2006,

showed that a concentration of 5 X $10^{-4}$ M of caulilexin C can inhibit 100% and 77% of the mycelial growth of *R. solani* and *L. maculans*, respectively. The relatively high efficacy of caulilexin C against *L. maculans* allows us to speculate that the significant increase of caulilexin C concentration observed in our study in DH378 at 14 dpi in comparison with the susceptible cultivar Lorenz might contribute towards factors that make DH387 perform better against *L. maculans* infections compared to Lorenz. Phytoanticipins were often considered among the mechanisms contributing to quantitative resistance (Poland et al. 2009; Kushalappa et al. 2016).

In general, *L. maculans* is capable of metabolising the chemical group indolyl-3-acetonitriles, such as caulilexin C, and it gives the respective indole-3-carboxylic acids that are not toxic to *L. maculans* as a chemical group (Pedras and Abdoli, 2017). Detoxification of caulilexin C gives a 1-methoxyindole-3-carboxylic acid (Pedras and Hossain, 2011). In our study, it was observed that phoma lesions at the stem base of DH378 did not expand after 14 dpi and the level of caulilexin C decreased. In contrast, the phoma lesions and the accumulation of caulilexin C continued after 14 dpi in the susceptible Lorenz. The decreased concentration after 14 dpi in DH378 might be due to the degradation of caulilexin C when the infection pressure decreased unlike with Lorenz where the accumulation was sustained with the intensified pathogen invasion.

*B. napus* showed to produce phytoalexins not only when infected with *L. maculans* as in our study, but also when infected with *V. longisporum* (Su, 2020), with *P. brassicae* (Pedras et al. 2008c) or with the oomycete *Albugo candida* (Pedras et al. 2008b). However, the detection timepoint of the phytoalexins may vary among different pathosystems. For instance, cyclobrassinin and rutalexin were detected in the roots of oilseed rape infected with *P. brassicae* after three and four weeks post inoculation, respectively. In a similar pathosystem, both spirobrassinin and brassilexin were only sensed five weeks post inoculation (Pedras et al, 2008c). In our study, all phytoalexins were observed one week after inoculation.

Brassilexin showed the lowest concentration among the phytoalexins accumulated in *B. napus* stems infected with *L. maculans* in this experiment. This was not surprising, as it is well known that high brassilexin accumulation in *Brassicae* species is often strongly correlated to the presence of the B genom. Rouxel et al. 1990 showed that brassilexin was produced in high concentrations in cotyledons of *B. juncea*, *B. carinata*, and *B. nigra*, while in very low concentrations in *B. napus* after exposure to $CuCl_2$.

*L. maculans* can detoxify brassilexin, at the first step to 3-aminomethyleneindole-2-thione, then to the final product 3-formylindolyl-2-sulfonic acid (Pedras and Suchy, 2005). This partially explains the decreased brassilexin concentration in both the susceptible and resistant genotypes at 14 dpi in our study. However, the reason for the further increase of brassilexin concentration at 21 dpi is not clear.

Rutalexin was first described in the tubers of the crucifer rutabaga (*B. napus L. ssp. rapifera)* (Pedras et al. 2004). In this experiment, rutalexin accumulated in high concentrations - almost similar to spirobrassinin - at 7 dpi in oilseed rape stems. Also, it was significantly more produced in the resistant genotype DH378 compared to the susceptible cultivar Lorenz. However, it is difficult to speculate about its role in resistance. That is mainly because rutalexin antifungal effect against *L. maculans* has not yet been investigated since rutalexin was not soluble in the culture media on which *L. maculans* can be cultivated in vitro (Pedras et al. 2004).

In our study, spirobrassinin had the highest concentration among the phytoalexins investigated across all timepoints while cyclobrassinin was ranked as the second most occuring phytoalexin at 14 and 21 dpi. This contradicts the findings of Pedras et al (2008c), which showed that cyclobrassinin was the highest produced phytoalexin in the roots of oilseed rape facing *P.* brassicae (Pedras et al. 2008c). This difference bewteen the two pathosystems could be illustrated by the detoxification efficacy of the two pathogens to each phytoalexin (Pedras and Abdoli, 2017).

Cyclobrassinin, the second-most dominant phytoalexin in the *L. maculans- B. napus* pathosystem, was significantly higher in concentration in the resistant genotype compared to the susceptible one, and its concentration decreased insignificantly over time. Although *L. maculans* can detoxify cyclobrassinin, its high concentration in this pathosystem could be explained by the synergistic interaction of brassilexin and cyclobrassinin - namely the inhibitory effect of brassilexin on the detoxification enzymes of cyclobrassinin hydrolase (Pedras and Minic, 2014). The presence of brassilexin may contribute to the relatively high concentration of cyclobrassinin. Future studies could investigate the role of brassilexin as a paldoxin.

All in all, despite the wide spectrum of plant defence chemicals produced in reaction to biotic stresses, we investigated here selected compounds that were described in the literature to present a reasonable concentration in *B. napus* exposed to biotic stressors (Pedras et al. 2008c). We showed the dynamics of their induction over time in oilseed rape infected with *L. maculans* in the proximity of the infection site. This approach can be especially helpful to study phytoalexins correlated to quantitative resistance depicted in stems of *B. napus* infected

with *L. maculans* after ensuring that these phytoalexins remain under the limits of detection by injured mock-plants. It is premature to propose that the investigated compounds in this study play causal roles in quantitative resistance. However, further investigations should focus on genetic studies to define genes related to the compounds that correlated with resistance. This can help determine whether they are key factors for quantitative resistance mechanisms that help cope with *L. maculans* infections.

**Acknowledgement**

This work was financially supported by the German Federal Ministry for Food and Agriculture (BMEL). The authors acknowledge guidance provided by Dr. Anna Rathgeb with the HPLC analysis of the preliminary samples.

**Author contribution**

Birger Koopmann, Petr Karlovsky and Dima Alnajar designed the research. Dima Alnajar conducted the green house experiment and secondary metabolite extraction. Dima Alnajar and Mohammad Alhussein prepared the HPLC samples and conducted the analysis. Dima Alnajar wrote the manuscript. Andreas von Tiedemann and Birger Koopmann supervised the work and reviewed the manuscript.

**References**

Agrios, G.N. 2005. Plant pathology. Elsevier Academic Press, Amsterdam, Netherlands, 5th ed, pp.125, ISBN 978-0-12-044565-3

Ahuja, I., Kissen, R. and Bones, AM. 2012. Phytoalexins in defense against pathogens. *Trends Plant Sci.* 17:73–90. doi:10.1016/j.tplants.2011.11.002.

Andréasson, E., Bolt Jorgensen, L., Höglund, AS., Rask, L. and Meijer, J. 2001. Different myrosinase and idioblast distribution in *Arabidopsis* and *Brassica napus*. *Plant Physiol.* 127:1750–1763. doi:10.1104/pp.010334.

Bigeard, J., Colcombet, J., and Hirt, H. 2015. Signaling mechanisms in pattern-triggered immunity (PTI). *Mol Plant* 8: 521–539. doi:10.1016/j.molp.2014.12.022.

Dahiya, JS. and Rimmer, S. 1988. Phytoalexin accumulation in tissues of *Brassica napus* inoculated with *Leptosphaeria maculans*. *Phytochemistry*. 27:3105–3107. doi:10.1016/0031-9422(88)80009-8.

Fitt, BDL., Brun, H., Barbetti, MJ. and Rimmer, SR. 2006. Worldwide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *Eur. J. Plant Pathol.* 114:3–15. doi:10.1007/s10658-005-2233-5.

French, E., Kim, B.S., and Iyer-Pascuzzi, A.S. 2016. Mechanisms of quantitative disease resistance in plants. *Semin. Cell Dev. Biol.* 56: 201–208. doi:10.1016/j.semcdb.2016.05.015.

Fukuoka, S., Yamamoto, S. I., Mizobuchi, R., Yamanouchi, U., Ono, K., Kitazawa, N., Yasuda, N., Fujita, Y., Thi Thanh Nguyen, T., Koizumi, S., Sugimoto, K., Matsumoto, T., and Yano, M. 2015. Multiple functional polymorphisms in a single disease resistance gene in rice enhance durable resistance to blast. *Sci. Rep.* 4: 4550. doi:10.1038/srep04550.

Hartmann, T. 2008. The lost origin of chemical ecology in the late 19th century. *Proc. Natl. Acad. Sci. U S A.* 105:4541–4546. doi:10.1073/pnas.0709231105.

Hasegawa, M., Mitsuhara, I., Seo, S., Imai, T., Koga, J., Okada, K., Yamane, H. and Ohashi, Y. 2010. Phytoalexin accumulation in the interaction between rice and the blast fungus. *Mol. Plant Microbe Interact.* 23:1000–1011. eng. doi:10.1094/MPMI-23-8-1000.

He, X.Z., and Dixon, R.A. 2000. Genetic manipulation of Isoflavone 7- *O* -Methyltransferase enhances biosynthesis of 4′-O-Methylated Isoflavonoid phytoalexins and disease resistance in Alfalfa. *Plant Cell* 12: 1689–1702. doi:10.1105/tpc.12.9.1689.

Heitefuss, R. 1982. Active defense mechanisms in plants. Plenum press : In cooperation with NATO Scientific Affairs Division, New York. URL: http://catalog.hathitrust.org/api/volumes/oclc/7835819.html, accessed on 20.01. 2022.

Hipskind, JD. and Paiva, NL. 2000. Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis*. *Mol. Plant Microbe Interact.* 13:551–562. doi:10.1094/MPMI.2000.13.5.551.

Ibraheem, F., Gaffoor, I. and Chopra, S. 2010. Flavonoid phytoalexin-dependent resistance to anthracnose leaf blight requires a functional yellow seed1 in *Sorghum bicolor*. *Genetics* 184:915–926. doi:10.1534/genetics.109.111831.

Jeandet, P., Clément, C., Courot, E. and Cordelier, S. 2013. Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. *Int. J. Mol. Sci.* 14:14136–14170. doi:10.3390/ijms140714136.

Kushalappa, AC., Yogendra, KN. and Karre, S. 2016. Plant innate immune response: qualitative and quantitative resistance. *Crit. Rev. Plant Sci.* 35:38–55. doi:10.1080/07352689.2016.1148980.

Kutcher, H.R., van den Berg, C.G.J., and Rimmer, S.R. 1993. Variation in pathogenicity of *Leptosphaeria maculans* on *Brassica* spp. based on cotyledon and stem reactions. *Can. J. Plant Pathol.* 15: 253–258. doi:10.1080/07060669309501920.

Li, Z. K., Luo, L.J., Mei, H.W., Paterson, A.H., Zhao, X.H., Zhong, D.B., Wang, Y.P., Yu, X.Q., Zhu, L., Tabien, R., Stansel, J.W., and Ying, C.S. 1999. A “defeated” rice resistance gene acts as a QTL against a virulent strain of *Xanthomonas oryzae pv. oryzae*. *Mol. Gen. Genet.* 261: 58–63. doi:10.1007/s004380050941.

Madden, L.V., Hughes, G., and van den Bosch, F. 2017. Temporal analysis I: quantifying and comparing epidemics. In: Madden, L.V., Hughes, G., and van den Bosch, 2017. The study of plant disease epidemics, APS, 63-116. doi:10.1094/9780890545058. ISBN:978-0-89054-505-8.

Newman PL. 1984. Differential host-parasite interactions between oilseed rape and *Leptosphaeria macufans*, the causal fungus of stem canker. *Plant Pathol.* 33:205–210. doi:10.1111/j.1365-3059.1984.tb02641.x.

Pedras M.S.C. and Abdoli A. 2017. Pathogen inactivation of cruciferous phytoalexins: detoxification reactions, enzymes and inhibitors. *RSC Adv.* 7:23633–23646. doi:10.1039/C7RA01574G.

Pedras M.S.C., Abdoli A. and Sarma-Mamillapalle VK. 2017. Inhibitors of the detoxifying enzyme of the phytoalexin brassinin based on quinoline and isoquinoline scaffolds. *Molecules.* 22(8):1345. doi:10.3390/molecules22081345.

Pedras M.S.C., Alavi M. and To QH. 2015. Expanding the nasturlexin family: nasturlexins C and D and their sulfoxides are phytoalexins of the crucifers *Barbarea vulgaris* and *B. verna*. *Phytochemistry*. 118:131–138. doi:10.1016/j.phytochem.2015.08.009.

Pedras M.S.C. and To QH. 2015. Non-indolyl cruciferous phytoalexins: Nasturlexins and tridentatols, a striking convergent evolution of defenses in terrestrial plants and marine animals? *Phytochemistry.* 113:57–63. doi:10.1016/j.phytochem.2014.07.024.

Pedras, M.S.C. and Yaya, E.E. 2015. Plant chemical defenses: are all constitutive antimicrobial metabolites phytoanticipins? *Nat. Prod. Commun.* 10: 1934578X1501000. doi:10.1177/1934578X1501000142

Pedras M.S.C. and Minic Z. 2014. The phytoalexins brassilexin and camalexin inhibit cyclobrassinin hydrolase, a unique enzyme from the fungal pathogen *Alternaria brassicicola*. *Bioorg. Med. Chem.* 22:459–467. doi:10.1016/j.bmc.2013.11.005.

Pedras M.S.C. and Yaya EE. 2014. Tenualexin, other phytoalexins and indole glucosinolates from wild cruciferous species. *Chem. Biodivers.* 11:910–918. doi:10.1002/cbdv.201300260.

Pedras M.S.C. and Sarma-Mamillapalle VK. 2012. The cruciferous phytoalexins rapalexin A, brussalexin A and erucalexin: chemistry and metabolism in *Leptosphaeria maculans*. *Bioorg. Med. Chem.* 20:3991–3996. doi:10.1016/j.bmc.2012.05.020.

Pedras M.S.C, Yaya EE and Glawischnig E. 2011. The phytoalexins from cultivated and wild crucifers: chemistry and biology. *Nat. Prod. Rep.* 28:1381–1405. doi:10.1039/c1np00020a.

Pedras M.S.C. and Hossain S. 2011. Interaction of cruciferous phytoanticipins with plant fungal pathogens: indole glucosinolates are not metabolized but the corresponding desulfo-derivatives and nitriles are. *Phytochemistry.* 72:2308–2316. doi:10.1016/j.phytochem.2011.08.018.

Pedras, M.S.C., and Yaya, E.E. 2010. Phytoalexins from *Brassicaceae*: News from the front. *Phytochemistry* 71: 1191–1197. doi:10.1016/j.phytochem.2010.03.020.

Pedras M.S.C., Minic Z. and Sarma-Mamillapalle VK. 2009. Substrate specificity and inhibition of brassinin hydrolases, detoxifying enzymes from the plant pathogens *Leptosphaeria maculans* and *Alternaria brassicicola. FEBS J.* 276:7412–7428. eng. doi:10.1111/j.1742-4658.2009.07457.x.

Pedras M.S.C., Minic Z. and Jha M. 2008a. Brassinin oxidase, a fungal detoxifying enzyme to overcome a plant defense - purification, characterization and inhibition. *FEBS J.* 275:3691–3705. doi:10.1111/j.1742-4658.2008.06513.x.

Pedras M.S.C, Zheng Q.A., Gadagi R.S. and Rimmer S.R. 2008b. Phytoalexins and polar metabolites from the oilseeds canola and rapeseed: differential metabolic responses to the biotroph *Albugo candida* and to abiotic stress. *Phytochemistry.* 69:894–910. doi:10.1016/j.phytochem.2007.10.019.

Pedras M.S.C., Zheng Q.A. and Strelkov S. 2008c. Metabolic changes in roots of the oilseed canola infected with the biotroph *Plasmodiophora brassicae*: phytoalexins and phytoanticipins. *J. Agric. Food Chem.* 56:9949–9961. doi:10.1021/jf802192f.

Pedras M.S.C., Sarwar MG., Suchy M. and Adio AM. 2006. The phytoalexins from cauliflower, caulilexins A, B and C: isolation, structure determination, syntheses and antifungal activity. *Phytochemistry.* 67:1503–1509. doi:10.1016/j.phytochem.2006.05.020.

Pedras M.S.C. and Suchy M. 2005. Detoxification pathways of the phytoalexins brassilexin and sinalexin in *Leptosphaeria maculans*: isolation and synthesis of the elusive intermediate 3-formylindolyl-2-sulfonic acid. *Org. Biomol. Chem.* 3:2002–2007. doi:10.1039/B501907A.

Pedras, M.S.C., Montaut, S., and Suchy, M. 2004. Phytoalexins from the crucifer rutabaga: structures, syntheses, biosyntheses, and antifungal activity. *J. Org. Chem.* 69: 4471–4476. doi:10.1021/jo049648a.

Pedras M.S.C. and Khan AQ. 1996. Biotransformation of the *Brassica* phytoalexin brassicanal A by the blackleg fungus. *J Agric Food Chem.* 44:3403–3407. doi:10.1021/jf960098u.

Pedras M.S.C. and Taylor JL. 1993. Metabolism of the phytoalexin brassinin by the "blackleg" fungus. *J. Nat. Prod.* 56:731–738. doi:10.1021/np50095a010.

Persson M., Staal J., Oide S. and Dixelius C. 2009. Layers of defense responses to *Leptosphaeria maculans* below the RLM1- and camalexin-dependent resistances. *New Phytol.* 182:470–482. doi:10.1111/j.1469-8137.2009.02763.x.

Peyraud, R., Dubiella, U., Barbacci, A., Genin, S., Raffaele, S., and Roby, D. 2017. Advances on plant-pathogen interactions from molecular toward systems biology perspectives. *Plant J.* 90: 720–737. doi:10.1111/tpj.13429.

Poland J.A., Balint-Kurti P.J., Wisser R.J., Pratt R.C. and Nelson R.J. 2009. Shades of gray: the world of quantitative disease resistance. *Trends Plant Sci.* 14:21–29. doi:10.1016/j.tplants.2008.10.006.

Raman, H., Raman, R., Coombes, N., Song, J., Diffey, S., Kilian, A., Lindbeck, K., Barbulescu, D.M., Batley, J., Edwards, D., Salisbury, P.A., and Marcroft, S. 2016. Genome-wide association study identifies new loci for resistance to *Leptosphaeria maculans* in canola. *Front. Plant Sci.* 7:1513. doi:10.3389/fpls.2016.01513.

Raman, H., Raman, R., Diffey, S., Qiu, Y., McVittie, B., Barbulescu, D.M., Salisbury, P.A., Marcroft, S., and Delourme, R. 2018. Stable quantitative resistance loci to blackleg disease in Canola (*Brassica napus* L.) over continents. *Front. Plant Sci.* 9: 1622. doi:10.3389/fpls.2018.01622.

Rouxel T. and Balesdent M.H. 2005. The stem canker (blackleg) fungus, *Leptosphaeria maculans*, enters the genomic era. *Mol. Plant Pathol.* 6:225–241. doi:10.1111/j.1364-3703.2005.00282.x.

Rouxel, T., Kollmann, A., Boulidard, L., and Mithen, R. 1991. Abiotic elicitation of indole phytoalexins and resistance to *Leptosphaeria maculans* within *Brassiceae*. *Planta* 184: 271–278. doi:10.1007/BF00197957.

Rouxel T., Renard M., Kollmann A. and Bousquet J.F. 1990. Brassilexin accumulation and resistance to *Leptosphaeria maculans* in *Brassica* spp. and progeny of an interspecific cross *B. juncea* × *B. napus*. *Euphytica.* 46:175–181. doi:10.1007/BF00022313.

Su, L., Rathgeb A., Figueroa López C. and Karlovsky P. 2020. Induction of phytoalexins synthesis in oilseed rape by *Verticillium longisporum* and their detoxification by fungi. In: Su L. 2020. Fungal bioactive molecules in interactions with plant and animals. Georg-August-University Göttingen, Göttingen, Germany. pp:81-118. URL: https://ediss.uni-goettingen.de/handle/21.11130/00-1735-0000-0005-138D-7?locale-attribute=en. Accessed on: 02.05.2021.

# Chapter VII: General discussion

Global demand for *B. napus* is continuously increasing, as this oleaginous crop is one of the most important sources of biodiesel. With the rise of oilseed prices, the cultivated area of oilseed rape has increased to 6 million hectares in Europe´s top ten producing countries, as reported in January 2022 (European Seed, 2022). However, this does not translate into increased production, not only due to the violent conflicts in the growing regions, but also because productivity is related to the ability to manage biotic and abiotic stresses that considerably limit yields (Elferjani and Soolanayakanahally, 2018; Zheng et al. 2020). Sharp changes in weather conditions in the last few years have affected yield directly through abiotic stresses. They also provided better conditions for a spectrum of pest and diseases to thrive. Phoma stem canker is an example, as it was observed that a warm and wet autumn in Europe has elevated yield losses attributed to *L. maculans* infections (Brachaczek et al. 2021).

*L. maculans* can be managed based on an integrated approach that combines cultural methods, pesticide applications and growing resistant cultivars. Using resistant cultivars is known to be the most sustainable and effective blackleg disease control method. Yet, applying other control methods plays an important role in mitigating the disease, and it is indispensable to extend the durability of resistance resources (Aubertot et al. 2006). Conventional tillage helps to manage infected stubble, which limits the establishment of *L. maculans* inoculum in the respective field. However, it does not protect oilseed rape plants from wind-borne ascospores from neighboring fields. Besides, the speed of stubble decomposition is related to the weather conditions in the region and to the microorganism spectrum in the soil (Ash 2000; Naseri et al. 2008; Brachaczek et al. 2021). Early fungicide treatments of a susceptible oilseed rape cultivar against blackleg disease can significantly increase seed yield under medium to severe infection pressure (Peng et al. 2020). Applying fungicides against blackleg disease is often carried out several times per season. The cost of applying several treatments can be economically justified in severe blackleg infection cases when conditions are warm (> 60°C cumulative mean temperature) and wet (> 110 mm rainfall) between BBCH 14-BBCH19 (Brachaczek et al. 2021). In Australia, chemical applications are the main control method of *L. maculans* (Van de Wouw et al. 2021). The ban of several pesticides in the European Union among them some that target blackleg disease, such as flusilazole (Brachaczek et al. 2021) have led to increased efforts in searching for genetic resistance that can be deployed in new cultivars to control blackleg disease. This study is a contribution towards these efforts. Here, we characterized the causal agent of blackleg disease and defined the efficacy of major *R* genes in German fields. We sought new resources for quantitative resistance, the more

durable resistance to blackleg disease. Additionally, we studied a spectrum of phytoalexins and phytoanticipins in relation to blackleg disease resistance.

Depending on resistance type, resistant cultivars to blackleg infection show immunity at the beginning or late in the season. Functional major *R* genes are mainly expressed at cotyledon and first leaves stages, leading to hypersensitive reactions that stop disease development. On the contrary, adult resistance - conditioned principally by QTLs - mitigates *L. maculans* infection at the stem level during the adult stage, although plant leaves might show considerable phoma spots throughout autumn and spring (Amas et al. 2021). Many factors have pushed research efforts towards a focus on major *R* genes over the last two decades (Yu et al. 2005; Long et al. 2011; Larkan et al. 2013; Yu et al. 2013; Raman et al. 2021). The healthy appearance of cultivars harboring major *R* genes at the beginning of the season relieve oilseed rape growers, and thus, they favor such cultivars. Additionally, it is more feasible to launch breeding programs that deal with fewer major *R* genes than with quantitative disease resistance (QDR), which is controlled by many genes (Poland et al. 2009). A disadvantage of major *R* genes is that they go through a cycle of boom and bust, which may lead them to turn ineffective a few years after commercial release. Consequently, regular monitoring of changes in the pathogen population to define the actual efficacy of a major *R* gene in a certain region is needed to avoid sudden yield declines (McDonald and Linde 2002).

In this study, the race spectrum of *L. maculans* was monitored in northern and central Germany to help oilseed rape growers decide which major *R* genes are likely to perform best in their fields in accordance with the pathogen population. However, this knowledge can only be put into practice if producers disclose the major *R* genes that have been crossed into commercially available varieties (Marcroft et al. 2012; Cornelsen et al. 2021). Another solution could be that academic institutions develop a tool, namely a *L. maculans* differential set, that tests new cultivars and identifies major *R* genes in commercial cultivars. Australia was a forerunner to officially apply a system for the strategic deployment of major *R* genes through establishing a scheme that helps growers apply major *R* gene rotations. In this system, major *R* genes are assigned to groups and commercial cultivars are given a label accordingly. Growers are recommended to annually use cultivars from different groups (Van de Wouw and Marcroft, 2012). In 2017, a similar system was established in Canada. The feasibility of the system was validated in the most important *B. napus* growing areas, the Canadian prairies (Cornelsen et al. 2021). In Germany, the chamber of agriculture, which supports growers with recommendations regarding commercial cultivars, describes the resistance level of oilseed rape cultivars to blackleg without mentioning the type of resistance, i.e. whether it is qualitative or quantitative, and without announcing the major *R* genes. For example, Lower Saxony’s

chamber of agriculture gives cultivar resistance three levels: under average, average, and above average (Landwirtschaftskammer Niedersachsen, 2021). This information that reflects cultivar tolerance to blackleg infection is essential and was not given up in the deployment systems of Canada and Australia (Van de Wouw and Marcroft, 2012, Cornelsen et al. 2021). Nevertheless, it is recommended to take steps that allow for the development of a strategic deployment scheme of major *R* genes in Germany, combining it with disease tolerance level descriptions. This study provides an update for the *Avr*-profile of *L. maculans* populations in several regions throughout Germany. It was clear from our results that the abundance of a specific avirulence gene might change from region to region, as shown in chapter II. To facilitate annual field-specific information for oilseed rape growers, commercial labs in Canada start to provide characterization services for *L. maculans* from samples sent by the famers (Cornelsen et al. 2021).

Although our study demonstrated regional differences in major *R* gene efficacies across Germany, the relative similarities enabled us to assign major *R* genes to categories: namely ineffective, partially effective and effective (chapter II). For example, *Rlm2* was ineffective in all fields investigated. In contrast, both *LepR2 and Rlm7* were effective in Germany despite the regional differences. These relative similarities can be explained by the fact that *L. maculans* ascorpores are wind-borne and the new virulent isolates can transfer spatially from one field to another (Fitt et al. 2006). Thus, isolation distance between oilseed rape fields seems to be crucial to control blackleg disease, as already recommended in previous studies (Travadon et al. 2011; Hossard et al. 2018). Isolation distance can be optimally applied by growing different crops in adjacent fields. Yet, growing different oilseed rape cultivars can also help to prolong the efficacy of major *R* genes. This might not be easy to apply as it needs to be synchronized between different farms through a regional strategic plan. The lack of diversity monoculture system provide is controversial feature of modern agriculture. On the one hand, growing one crop and one cultivar allows farmers to use specific machinery to effectively conduct agronomic services across large farms. On the other hand, it considerably increases the risk of pest and disease outbreaks due to the simplification of the cropping systems (Stoate et al. 2001; Balogh 2021).

Despite the progress achieved in defining avirulence genes and the corresponding major *R* genes, some confusion about explaining some *Avr-R* interactions has taken time to be explained - some are still to be reconciled. For instance, *Avrlm4* and *Avrlm7* were found to be alleles of one gene and were then renamed as *Avrlm4-7* (Parlange et al. 2009). A similar case was applied to *Avrlm5* and *Avrlm9*, which were renamed *Avrlm5-9* (Ghanbarnia et al. 2018). Regarding *AvrlepR3*, some studies suggest that *AvrlepR3* is an allele of *Avrlm1* and others

that *AvrLepR3 is as* the same as *Avrlm1.* It was also speculated that *AvrlepR3* is just a hypothetical gene (Dolatabadian et al. 2021). Although chapter II proved that many isolates were indeed *avrlm1AvrlepR3*, i.e. virulent on *Rlm1* and avirulent on *LepR3*, further studies are needed to confirm the genotype of such isolates and to prove the presence of *AvrlepR3* independently from *Avrlm1.* This may contribute to better understanding its interaction with *LepR3* and *Rlm1*.

This work focused as well on finding resources for QDR (chapter III, IV and V). The genetic variation in the gene pool of *B. napus* has significantly decreased with time due to the selection for quality features, especially the selection for minimum erucic acid content and low glucosinolate to produce the double-low varieties. Consequently, seeking new resistance resources has become more challenging (Friedt et al. 2018). One distinct aspect of this work was the plant material itself in which we sought for blackleg disease resistance in *B. napus*. On the one hand, an interconnected multiparent population that has a broad genetic background of seven different parents was phenotyped for resistance and relevant QTLs were identified (chapter III and IV). On the other hand, the project identified *L. maculans* resistance source expressed in *B. nigra,* a relative species of *B. napus,* and confirmed the ability to transfer these traits to *B. napus* (chapter V). Our phytopathological study showed that these plant materials have deployment potential that need to be researched further.

The gradual nature of QDR complicates its studies (Poland et al. 2009). Nevertheless, the larger the population, the higher the precision of the results and the better the genetic gain of a breeding program (Araus et al. 2018). In this study, we phenotyped a large interconnected multiparent mapping population of 354 DH lines under greenhouse and field conditions. This required a large team of assessors to visually assess blackleg symptoms. Visual assessments of large-scale trials is laborious and time consuming, but a critical disadvantage of it is the increased error related to the necessity of involving several assessors (Aubertot et al., 2004). Digital tools can accelerate phenotyping for resistance and reduce the assessor error. Such tools have recently become available. For instance, the use of multispectral 3D scanners for phenotyping under controlled conditions (e.g. Phenospex Smart Plant Analysis, 2022). Hyperspectral imaging was applied to differentiate QTLs related to resistance against Cercospora leaf spot in sugar beet. It proved to be a promising method for fast and accurate phenotyping under controlled conditions (Leucker et al. 2017). Sensor devices, such the handheld Trimble GreenSeeker® crop sensor, were successfully used in field trials to investigate QTLs related to resistance against wheat stripe rust (Pretorius et al. 2017). Kuska et al. (2017) used multispectral imaging to characterize powdery mildew (*Blumeria graminis*) interaction types with barley in the early stages of infection. The most successful cases

documented in literature about digital phenotyping were assigned to foliar diseases. In blackleg disease, phenotyping - depending on the assessment of the inner necrosis and the depth of the lesion - seems to be difficult to digitalise, although still worth examining. For field trials, it is worth investigating the feasibility of computer vision tools in trials where blackleg infections are sever and the phenotyping method depends on the survival of the plant (i.e. plant died vs. plant alive phenotyping).

Phenotyping for QDR under field conditions is important to ensure the stability of a QTL in diverse environments (Amas et al. 2021). However, field phenotyping trial failures are expected when disease pressure does not reach a threshold level that allow symptom phenoytping – this occurred in our study in season 2018/2019. Together with the suboptimal environmental conditions for *L. maculans* growth, failed infection might be due to changes that occurred within the plants, as their defence system against heat and drought stress could be activated early in the season. It can be speculated that priming the plant for abiotic stresses might contribute to stop the disease particularly as disease pressure was very low. For example, in spring, Peine and Sörup had 8% and 1% disease incidence, respectively. However, the disease development was slow and canker at the stem base was not formed. Abiotic stresses result in molecular, chemical, and physiological changes, such as fluctuations in plant cell pH, changes in leaf osmotic potential, relative water content, and nutritional imbalance (Yadav et al. 2020). Crosstalk between pathways responsible for abiotic and biotic resistance has been described in several cases (Tortosa et al. 2019, Sucher et al. 2020).

In chapter V, our phenotyping results were deployed in a genome-wide association study that identified eight QTLs on chromosomes A07 and A09 with significant small effects that could explain 3-6% of the phenotypic variation. Surprisingly, a novel variant of *Rlm9* found to be located within a detected QTL region on A07. Based on that, it was suggested that either the region described is a genetic hot spot for gene linkages responsible for qualitative and quantitative resistance, or that some variant of *Rlm9* may play a role in quantitative resistance (Vollrath et al. 2021). A similar case was described for *Rlm13,* which was found to be co-located within described QTLs (Raman et al. 2021). Further studies should test the function of the detected variants of *Rlm9* using gene editing approaches. These results underline the need to review the definitions of the two extreme types of resistance and allow us to assume a shade of grey between them (Poland et al. 2009). Many genes proved to be exceptions from classic definitions. For instance, *Rlm13* is a major *R* gene that is non-race specific (Raman et al. 2021). Likewise, *LepR2* showed resistance at both the cotyledon and stem base level (Xiang Neik et al. 2022). Also, resistance at cotyledon level does not mean that the decreased symptoms are exclusively ruled by major *R* genes but also quantitative resistance can

contribute to this resistance (Hubbard et al. 2020). Additionally, Stotz et al. (2014) suggested that neither pattern-triggered immunity (PTI) nor effector-triggered immunity (ETI) accurately describe several interactions between the apoplastic pathogen *L. maculans* with major *R* genes. Unlike ETI, in the *B. napus* - *L. maculans* interaction, pathogen detection does not occur intracellularly, but rather extracellularly through surface-localised receptor proteins. Another discrepancy to ETI is that the pathogen remains an endophyte for a couple of days before triggering a hypersensitive reaction. Thus, the term effector-triggered defence (ETD) was proposed (Stotz et al. 2014). However, the term has not been widely adopted in literature describing resistance in this pathosystem.

*B. napus* is a relatively new crop that has no wild type since it is an interspecific cross of two *Brassica* species: *B. oleracea* and *B. rapa.* Therefore, the genetic diversity in *B. napus* population is limited. Breeding for double-low cultivars further decreased the diversity in *B. napus* (Chalhoub et al. 2014). Introgressions have not only been used to expand the genetic base of the *B. napus* gene pool, but also to discover new genes that had not been described (Pratap et al. 2021). In *B. napus*, most major *R* genes discovered were located in the A genome (Delourme et al. 2006), while only *Rlm13* has been found in the C genome (Raman et al. 2021). All *Brassica* species that harbor the B genome, namely *B. nigra*, *B. juncea* and *B. carinata* are known to be resistant to *L. maculans* under field conditions (Rimmer and van den Berg, 1992). In our study, we showed that resistance to *L. maculans* can be introgressed into *B. napus* from *B. nigra*. Yet, the resulting allohexaploids were likely unstable as reported by our cooperation partners from the University of Giessen. Further studies to overcome this obstacle can be a quantum leap to allow the use of these allohexaploid for sound agronomic benefits.

The B genome of cruciferous plants was often associated with important agronomic traits. Ethiopian mustard (*B. carinata*) shows a high level of resistance to pod shatter (Raman et al. 2017). Resistance genes for *L. maculans* were introgressed from the B genome of *B. juncea* (Chèvre et al. 1997). Furthermore, the phytoalexin brassilexin (investigated in chapter VI) is produced in substantially higher concentrations in *Brassica* species that carry the B genome compared to those that do not (Rouxel et al. 1990). In our study, we were not able to describe all characters of blackleg resistance expressed in the interspecific hybrid Ningyou7 × 1X7. Unlike the parents, the hybrids were only tested at stem level and not at cotyledon level. This was due to the development process of the plant materials. Hybrids were produced by conducting an embryo rescue followed by a colchicine treatment. Plantlets were delivered to the Plant Pathology and Crop Protection Division, Georg-August University Göttingen, after being produced in the Department of Plant Breeding, Justus Liebig University Giessen.

Resistance phenotyping at the stem level of the hybrids confirmed that resistance in Ningyou7 × 1X7 was due to the B genome, as the *B. nigra* parent was resistant at the cotyledon and stem level, while the *B. napus* parent Ningyou7 was susceptible at both levels. However, it is unclear if this resistance is ruled by a single gene or more, and if it is race-specific or non- race specific reaction. Such information is indispensable once this plant material is ready to be involved in cultivar development programmes. Exploitation of artificially generated allopolyploids was documented in other main crops. In wheat, nascent hexaploidy showed robust seedling growth, larger spikes and more tolerance to salinity compared to their parents (Li et al. 2015).

Plant defense systems have many layers of complexity (Andersen et al. 2018). In chapter VI, our study investigated the occurrence of phytoalexins in susceptible and resistant oilseed rape genotypes. We showed that rutalexin, spirobrassinin, cyclobrassinin, and brassilexin are phytoalexins in this pathosystem. Their induction increased upon *L. maculans* infection in the resistant cultivar more than in the susceptible one. On the contrary, caulilexin C was found in *B. napus* stems before the infection, and thus described as a phytoanticipin. Caulilexin C was also increasingly accumulated upon infection. There are plenty of studies that investigated phytoalexins in cruciferous plants (reviewed in Pedras and Yaya, 2010). Most phytoalexins related to resistance were indole alkaloids derived from either (S)-tryptophan, often with a sulfur atom derived from cysteine (Pedras et al. 2011; Pedras and Yaya 2014) or from (S)-phenylalanine (Pedras and To, 2015; Pedras et al. 2015). N'Guyen et al. (2021) suggested that indolic phytoalexins mainly disturb the mitochondrial functions in fungal cells. The first phytoalexin in crucifers was reported in 1986, and until now 54 phytoalexins have been described in this family (Pedras and Abdoli 2017). Phytoalexin spectra are similar among members of a given plant family or at least closely related precursors are utilized (Pedras and Yaya 2010; Ahuja et al. 2012). Despite similarities in phytoalexin spectra among a plant family, differences between species were confirmed in previous studies. For example, *B. napus* is not able to produce camalexin, an important phytoalexin of *A. thaliana* involved in resistance against *L. maculans* (Persson et al. 2009; Becker et al. 2017). Thus, it is indispensable to study phytoalexin induction in each specific pathosystem.

The profile of induced phytoalexins may differ not only based on species but also based on the elicitor type, plant variety, or ecotype (Pedras et al. 2008; Pedras and Yaya, 2010). For instance, Su et al. (2020) proved that *B. napus* produced 700 times more spirobrassinin and 400 times more brassilexin after being treated with the abiotic stressor $CuSo_4$ compared with the amount induced after being infected with *V. longisporum*. The role of the ecotype was

proved by Kagan and Hammerschmidt (2002) where differences in camalexin accumulation were observed by comparing 24 ecotypes of *A. thaliana* infected with *Alternaria brassicicola*.

Knowledge about phytoalexins provide many possibilities to develop crop protection tools. Firstly, novel pesticide ideas could be developed by identifying plant elicitors of phytoalexins. Ning et al. (2003) proposed the developed of a new fungicide against the fungal pathogen *Colletotrichum gloeosporioides*, which leads to orange damage around harvest and post-harvest. The fungicide was formulated based on the chemical synthesis of the oligosaccharide phytoalexin elicitor glucohexatose and was tested in orange gardens. It showed more efficacy than some commercial pesticides used against the pathogen (Ning et al. 2003). Secondly, phytoalexins per se can be synthesized as a fungicide. Yang et al. (2002) showed that analogues of flavanone, an effective phytoalexin against the pathogen *Magnaporthe grisea*, are suitable for fungicide development against rice blast. A third advantage of phytoalexin studies is the opportunity to synthesize paldoxins as crop protection tools. Paldoxins disfunction pathogens detoxify enzymes and prevent them from detoxifying phytoalexins. For instance, Pedras et al. (2017) synthesized several quinoline and isoquinoline scaffolds and demonstrated their ability to effectively inhibit the enzyme brassinin oxidase produced by *L. maculans* (BOLm). Finally, breeding programmes can introgress genes that might be proved responsible for the activation of phytoalexin synthesis pathway to develop new resistant cultivars.

Loss-of-function and gain-of-function approaches that target RNA, DNA or proteins were used extensively to confirm a causative relationship between phytoalexins induction and plant resistance. Using the loss-of-function technique, Ibraheem et al. (2010) showed that a functional yellow seed1 (y1) gene, which regulates key steps in the pathway of the phytoalexin 3-deoxyanthocyanidin production, is essential for *Sorghum bicolor* resistance to the leaf blight caused by *Colletotrichum sublineolum*. Also, the gain-of-function approach was examined to check the possibility of developing genetically modified resistant plants by inserting a gene that enhances phytoalexins production. For example, transgenic alfalfa plants were able to accumulate the phytoalexin resveratrol-3-O- beta-D- glucopyranoside. This acts as an inhibitor for the pathogen *Phoma medicaginis* (Hipskind and Paiva, 2000). Further studies should use these approaches to prove whether the phytoalexins correlated with resistance in our study have causative relationships to it as well. This helps to unravel the potential of these phytoalexins for the further exploitation as oilseed rape protection tool against blackleg.

Overall, this study characterized the pathogen *L. maculans* in German fields and showed the extent to which the currently available major *R* genes are still effective. The study showed that the efficacy of intensively deployed major *R* genes could be lost, but also provided

recommendations to improve their durability. Additionally, phenotyping results and the study on phytoalexins and phytoanticipins can be further used by researchers to improve new resistance cultivars or chemical control methods of blackleg disease in *B. napus*.

**References**

Ahuja, I., Kissen, R., and Bones, A.M. 2012. Phytoalexins in defense against pathogens. *Trends Plant Sci.* 17: 73–90. doi:10.1016/j.tplants.2011.11.002.

Amas, J., Anderson, R., Edwards, D., Cowling, W., and Batley, J. 2021. Status and advances in mining for blackleg (*Leptosphaeria maculans*) quantitative resistance (QR) in oilseed rape (*Brassica napus*). *Theor. Appl. Genet.* 134: 3123–3145. doi:10.1007/s00122-021-03877-0.

Andersen, E., Ali, S., Byamukama, E., Yen, Y., and Nepal, M. 2018. Disease resistance mechanisms in plants. *Genes* **9**: 339. doi:10.3390/genes9070339.

Araus, J.L., Kefauver, S.C., Zaman-Allah, M., Olsen, M.S., and Cairns, J.E. 2018. Translating high-throughput phenotyping into genetic gain. *Trends Plant Sci.* 23: 451–466. doi:10.1016/j.tplants.2018.02.001.

Ash, G. 2000. Blackleg of oilseed rape. *The Plant Health Instructor APS*. doi:10.1094/PHI-I-2000-1111-01.

Aubertot, J.-N., Schott, J.-J., Penaud, A., Brun, H., and Doré, T. 2004. Methods for sampling and assessment in relation to the spatial pattern of phoma stem canker (*Leptosphaeria maculans*) in oilseed rape. *Eur. J. Plant Pathol.* 110: 183–192. doi:10.1023/B:EJPP.0000015359.61910.3b.

Aubertot, J.N., West, J.S., Bousset-Vaslin, L., Salam, M.U., Barbetti, M.J., and Diggle, A.J. 2006. Improved resistance management for durable disease control: a case study of phoma stem canker of oilseed rape (*Brassica napus*). *Eur. J. Plant Pathol.* 114: 91–106. doi:10.1007/s10658-005-3628-z.

Balogh, A. 2021.December 13. The rise and fall of monoculture farming. URL: https://ec.europa.eu/research-and-innovation/en/horizon-magazine/rise-and-fall-monoculture-farming. Accessed on: 30.04.2022.

Becker, M.G., Zhang, X., Walker, P.L., Wan, J.C., Millar, J.L., Khan, D., Granger, M.J., Cavers, J.D., Chan, A.C., Fernando, D.W.G., and Belmonte, M.F. 2017. Transcriptome analysis of the *Brassica napus* - *Leptosphaeria maculans* pathosystem identifies receptor, signaling and structural genes underlying plant resistance. *Plant J.* 90: 573–586. doi:10.1111/tpj.13514.

Brachaczek, A., Kaczmarek, J., and Jedryczka, M. 2021. Warm and wet autumns favour yield losses of oilseed rape caused by phoma stem canker. *Agronomy* 11: 1171. doi:10.3390/agronomy11061171.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wang, X., Chiquet, J., Belcram, H., Tong, C., Samans, B., Corréa, M., Da Silva, C., Just, J., Falentin, C., Koh, C.S., Le Clainche, I., Bernard, M., Bento, P., Noel, … and Wincker, P. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science* 345: 950–953. doi:10.1126/science.1253435.

Chèvre, A.M., Barret, P., Eber, F., Dupuy, P., Brun, H., Tanguy, X., and Renard, M. 1997. Selection of stable *Brassica napus-B. juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*).1. Identification of molecular markers, chromosomal and genomic origin of the introgression. *Theor. Appl. Genet.* 95: 1104–1111. doi:10.1007/s001220050669.

Cornelsen, J., Zou, Z., Huang, S., Parks, P., Lange, R., Peng, G., and Fernando, W.G.D. 2021. Validating the strategic deployment of blackleg resistance gene groups in commercial canola fields on the Canadian prairies. *Front. Plant Sci.* 12: 669997. doi:10.3389/fpls.2021.669997.

Delourme, R., Chèvre, A.M., Brun, H., Rouxel, T., Balesdent, M.H., Dias, J.S., Salisbury, P., Renard, M., and Rimmer, S.R. 2006. Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *Eur. J. Plant Pathol.* 114: 41–52. doi:10.1007/s10658-005-2108-9.

Dolatabadian, A., Cornelsen, J., Huang, S., Zou, Z., and Fernando, W.G.D. 2021. Sustainability on the farm: breeding for resistance and management of major canola diseases in Canada contributing towards an IPM approach. *Can. J. Plant Pathol.* 1-34. doi:10.1080/07060661.2021.1991480.

Elferjani, R., and Soolanayakanahally, R. 2018. Canola responses to drought, heat, and combined stress: shared and specific effects on carbon assimilation, seed yield, and oil composition. *Front. Plant Sci.* 9: 1224. doi:10.3389/fpls.2018.01224.

European Seed 2022. High prices lead to increase in oilseed rape acreage across Europe. URL: https://european-seed.com/2022/03/high-prices-lead-to-increase-in-oilseed-rape-acreage-across-europe/. Accessed on: 20.06.2022.

Fitt, B.D.L., Brun, H., Barbetti, M.J., and Rimmer, S.R. 2006. World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *Eur. J. Plant. Pathol.* 114: 3–15. doi:10.1007/s10658-005-2233-5.

Friedt, W., Tu, J., and Fu, T. 2018. Academic and economic importance of *Brassica napus* rapeseed. In: S. Liu, R. Snowdon, and B. Chalhoub, eds. The *Brassica napus* genome. Springer International Publishing, Cham. P: 1-20. doi:10.1007/978-3-319-43694-4_1.

Ghanbarnia, K., Ma, L., Larkan, N.J., Haddadi, P., Fernando, W.G.D., and Borhan, M.H. 2018. *Leptosphaeria maculans AvrLm9*: a new player in the game of hide and seek with *AvrLm4-7*. Mol. Plant Pathol. 19: 1754–1764. doi:10.1111/mpp.12658.

Hipskind, J.D., and Paiva, N.L. 2000. Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis*. *MPMI* 13: 551–562. doi:10.1094/MPMI.2000.13.5.551.

Hossard, L., Souchere, V., and Jeuffroy, M.H. 2018. Effectiveness of field isolation distance, tillage practice, cultivar type and crop rotations in controlling phoma stem canker on oilseed rape. *Agric. Ecosyst. Environ.* 252: 30–41. doi:10.1016/j.agee.2017.10.001.

Hubbard, M., Zhai, C., and Peng, G. 2020. Exploring mechanisms of quantitative resistance to *Leptosphaeria maculans* (Blackleg) in the cotyledons of canola (*Brassica napus*) based on transcriptomic and microscopic analyses. *Plants* 9: 864. doi:10.3390/plants9070864.

Ibraheem, F., Gaffoor, I., and Chopra, S. 2010. Flavonoid phytoalexin-dependent resistance to anthracnose leaf blight requires a functional *yellow seed1* in *Sorghum bicolor*. *Genetics* 184: 915–926. doi:10.1534/genetics.109.111831.

Kagan, I.A., and Hammerschmidt, R. 2002. Arabidopsis ecotype variability in camalexin production and reaction to infection by *Alternaria brassicicola*. *J. Chem. Ecol.* 28: 2121–2140. doi:10.1023/A:1021020512846.

Landwirtschaftskammer Niedersachsen 2021. Ergebnisse der Landessortenversuche Winter raps 2021. URL: https://www.lwk-niedersachsen.de/lwk/news/38256_Landessortenversuche_2021_Winterraps. Accessed: 06.06.2022

Larkan, N.J., Lydiate, D.J., Parkin, I.A.P., Nelson, M.N., Epp, D.J., Cowling, W.A., Rimmer, S.R., and Borhan, M.H. 2013. The *Brassica napus* blackleg resistance gene *LepR3* encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector *Avrlm1*. *New Phytol.* 197: 595–605. doi:10.1111/nph.12043.

Leucker, M., Wahabzada, M., Kersting, K., Peter, M., Beyer, W., Steiner, U., Mahlein, A.-K., and Oerke, E.-C. 2017. Hyperspectral imaging reveals the effect of sugar beet quantitative trait loci on Cercospora leaf spot resistance. *Funct. Plant Biol.* 44: 1. doi:10.1071/FP16121.

Li, A., Geng, S., Zhang, L., Liu, D., and Mao, L. 2015. Making the bread: insights from newly synthesized allohexaploid wheat. *Mol. Plant* 8: 847–859. doi:10.1016/j.molp.2015.02.016.

Long, Y., Wang, Z., Sun, Z., Fernando, D.W.G., McVetty, P.B.E., and Li, G. 2011. Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola cultivar ‘Surpass 400.’ *Theor. Appl. Genet.* 122: 1223–1231. doi:10.1007/s00122-010-1526-z.

Marcroft, S.J., Van de Wouw, A.P., Salisbury, P.A., Potter, T.D., and Howlett, B.J. 2012. Effect of rotation of canola (*Brassica napus*) cultivars with different complements of blackleg

resistance genes on disease severity: Rotating resistance genes to blackleg in canola. Plant Pathology **61**: 934–944. doi:10.1111/j.1365-3059.2011.02580.x.

McDonald, B.A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* 40: 349–379. doi:10.1146/annurev.phyto.40.120501.101443.

Naseri, B., Davidson, J.A., and Scott, E.S. 2008. Survival of *Leptosphaeria maculans* and associated mycobiota on oilseed rape stubble buried in soil. *Plant Pathol.* 57: 280–289. doi:10.1111/j.1365-3059.2007.01768.x.

N'Guyen, G.Q., Raulo, R., Porquier, A., Iacomi, B., Pelletier, S., Renou, J.-P., Bataillé-Simoneau, N., Campion, C., Hamon, B., Kwasiborski, A., Colou, J., Benamar, A., Hudhomme, P., Macherel, D., Simoneau, P., and Guillemette, T. 2021. Responses of the necrotrophic fungus *Alternaria brassisicola* to the indolic phytoalexin brassinin. *Front. Plant Sci.* 11: 611643. doi:10.3389/fpls.2020.611643.

Ning, J., Kong, F., Lin, B., and Lei, H. 2003. Large-scale preparation of the phytoalexin elicitor glucohexatose and its application as a green pesticide. *J. Agric. Food Chem.* 51: 987–991. doi:10.1021/jf020683x.

Parlange, F., Daverdin, G., Fudal, I., Kuhn, M.-L., Balesdent, M.-H., Blaise, F., Grezes-Besset, B., and Rouxel, T. 2009. *Leptosphaeria maculans* avirulence gene *AvrLm4-7* confers a dual recognition specificity by the *Rlm4* and *Rlm7* resistance genes of oilseed rape, and circumvents *Rlm4* -mediated recognition through a single amino acid change. *Mol. Microbiol.* 71: 851–863. doi:10.1111/j.1365-2958.2008.06547.x.

Pedras, M., Abdoli, A., and Sarma-Mamillapalle, V. 2017. Inhibitors of the detoxifying enzyme of the phytoalexin brassinin based on quinoline and isoquinoline scaffolds. *Molecules* 22: 1345. doi:10.3390/molecules22081345.

Pedras, M.S.C., and Abdoli, A. 2017. Pathogen inactivation of cruciferous phytoalexins: detoxification reactions, enzymes and inhibitors. *RSC Adv.* 7: 23633–23646. doi:10.1039/C7RA01574G.

Pedras, M.S.C., Alavi, M., and To, Q.H. 2015. Expanding the nasturlexin family: nasturlexins C and D and their sulfoxides are phytoalexins of the crucifers Barbarea vulgaris and B. verna. *Phytochemistry* 118: 131–138. doi:10.1016/j.phytochem.2015.08.009.

Pedras, M.S.C., and To, Q.H. 2015. Non-indolyl cruciferous phytoalexins: nasturlexins and tridentatols, a striking convergent evolution of defenses in terrestrial plants and marine animals? *Phytochemistry* 113: 57–63. doi:10.1016/j.phytochem.2014.07.024.

Pedras, M.S.C., and Yaya, E.E. 2010. Phytoalexins from *Brassicaceae*: News from the front. *Phytochemistry* 71: 1191–1197. doi:10.1016/j.phytochem.2010.03.020.

Pedras, M.S.C., and Yaya, E.E. 2014. Tenualexin, other phytoalexins and indole glucosinolates from wild cruciferous species. *Chem. Biodivers.* 11: 910–918. doi:10.1002/cbdv.201300260.

Pedras, M.S.C., Yaya, E.E., and Glawischnig, E. 2011. The phytoalexins from cultivated and wild crucifers: Chemistry and biology. *Nat. Prod. Rep.* 28: 1381. doi:10.1039/c1np00020a.

Pedras, M.S.C., Zheng, Q.-A., Gadagi, R.S., and Rimmer, S.R. 2008. Phytoalexins and polar metabolites from the oilseeds canola and rapeseed: differential metabolic responses to the biotroph *Albugo candida* and to abiotic stress. *Phytochemistry* 69: 894–910. doi:10.1016/j.phytochem.2007.10.019.

Peng, G., Liu, X., McLaren, D.L., McGregor, L., and Yu, F. 2020. Seed treatment with the fungicide fluopyram limits cotyledon infection by *Leptosphaeria maculans* and reduces blackleg of canola. *Can. J. Plant Pathol.* 42: 480–492. doi:10.1080/07060661.2020.1725132.

Persson, M., Staal, J., Oide, S., and Dixelius, C. 2009. Layers of defense responses to *Leptosphaeria maculans* below the *RLM1* - and camalexin-dependent resistances. *New Phytol.* 182: 470–482. doi:10.1111/j.1469-8137.2009.02763.x.

Phenospex- Smart Plant Analysis 2022. Phenospex- Smart Plant Analysis. URL: https://phenospex.com/products/plant-phenotyping/planteye-f600-multispectral-3d-scanner-for-plants/?gclid=Cj0KCQjwzLCVBhD3ARIsAPKYTcRc4cJFf1rKgkUwnOpgN8emlIzicXTYCUYu9g0Js13jHFDYoldlLHQaAuu1EALw_wcB. Accessed on: 19. 06. 2022

Poland, J.A., Balint-Kurti, P.J., Wisser, R.J., Pratt, R.C., and Nelson, R.J. 2009. Shades of gray: the world of quantitative disease resistance. *Trends Plant Sci.* 14: 21–29. doi:10.1016/j.tplants.2008.10.006.

Pratap, A., Das, A., Kumar, S., and Gupta, S. 2021. Current perspectives on introgression breeding in food legumes. *Front. Plant Sci.* 11: 589189. doi:10.3389/fpls.2020.589189.

Pretorius, Z.A., Lan, C.X., Prins, R., Knight, V., McLaren, N.W., Singh, R.P., Bender, C.M., and Kloppers, F.J. 2017. Application of remote sensing to identify adult plant resistance loci to stripe rust in two bread wheat mapping populations. *Precis. Agric.* 18: 411–428. doi:10.1007/s11119-016-9461-x.

Raman, H., Raman, R., Qiu, Y., Zhang, Y., Batley, J., and Liu, S. 2021. The *Rlm13* Gene, a new player of *Brassica napus–Leptosphaeria maculans* interaction maps on chromosome C03 in canola. *Front. Plant Sci.* 12: 654604. doi:10.3389/fpls.2021.654604.

Raman, R., Qiu, Y., Coombes, N., Song, J., Kilian, A., and Raman, H. 2017. Molecular diversity analysis and genetic mapping of pod shatter resistance Loci in *Brassica carinata* L. *Front. Plant Sci.* 8: 1765. doi:10.3389/fpls.2017.01765.

Rimmer, S.R., and van den Berg, C.G.J. 1992. Resistance of oilseed *Brassica* spp. to blackleg caused by *Leptosphaeria maculans*. *Can. J. Plant Pathol.* 14: 56–66. doi:10.1080/07060669209500906.

Rouxel, T., Renard, M., Kollmann, A., and Bousquet, J.-F. 1990. Brassilexin accumulation and resistance to *Leptosphaeria maculans* in *Brassica* spp. and progeny of an interspecific cross *B. juncea* × *B. napus. Euphytica* 46: 175–181. doi:10.1007/BF00022313.

Stoate, C., Boatman, N.D., Borralho, R.J., Carvalho, C.R., Snoo, G.R. de, and Eden, P. 2001. Ecological impacts of arable intensification in Europe. *J. Environ. Manage.* 63: 337–365. doi:10.1006/jema.2001.0473.

Stotz, H.U., Mitrousia, G.K., de Wit, P.J.G.M., and Fitt, B.D.L. 2014. Effector-triggered defence against apoplastic fungal pathogens. *Trends Plant Sci.* 19: 491–500. doi:10.1016/j.tplants.2014.04.009.

Su, L., Rathgeb A., Figueroa López C. and Karlovsky P. 2020. Induction of phytoalexins synthesis in oilseed rape by *Verticillium longisporum* and their detoxification by fungi. In: Su L. 2020. Fungal bioactive molecules in interactions with plant and animals. Georg-August-University Göttingen, Göttingen, Germany. pp:81-118. URL: https://ediss.uni-goettingen.de/handle/21.11130/00-1735-0000-0005-138D-7?locale-attribute=en. Accessed on: 02.05.2021.

Sucher, J., Mbengue, M., Dresen, A., Barascud, M., Didelon, M., Barbacci, A., and Raffaele, S. 2020. Phylotranscriptomics of the pentapetalae reveals frequent regulatory variation in plant local responses to the fungal pathogen *Sclerotinia sclerotiorum. Plant Cell* 32: 1820–1844. doi:10.1105/tpc.19.00806.

Tortosa, M., Cartea, M.E., Velasco, P., Soengas, P., and Rodriguez, V.M. 2019. Calcium-signaling proteins mediate the plant transcriptomic response during a well-established *Xanthomonas campestris* pv. *campestris* infection. *Hortic. Res.* 6: 103. doi:10.1038/s41438-019-0186-7.

Travadon, R., Sache, I., Dutech, C., Stachowiak, A., Marquer, B., and Bousset, L. 2011. Absence of isolation by distance patterns at the regional scale in the fungal plant pathogen *Leptosphaeria maculans. Fungal Biol.* 115: 649–659. doi:10.1016/j.funbio.2011.03.009.

Van de Wouw, A., and Marcroft, S. 2012. Classifying cultivars into different resistance groups. URL : http://www.australianoilseeds.com/__data/assets/pdf_file/0010/8659/8_VandeWouw_Marcroft_-_SABL.pdf. Accessed on: 06. 06. 2022

Van de Wouw, A.P., Marcroft, S.J., Sprague, S.J., Scanlan, J.L., Vesk, P.A., and Idnurm, A. 2021. Epidemiology and management of blackleg of canola in response to changing farming practices in Australia. *Australas. Plant Pathol.* 50: 137–149. doi:10.1007/s13313-020-00767-9.

Vollrath, P., Chawla, H.S., Alnajar, D., Gabur, I., Lee, H., Weber, S., Ehrig, L., Koopmann, B., Snowdon, R.J., and Obermeier, C. 2021. Dissection of quantitative blackleg resistance reveals novel variants of resistance gene *Rlm9* in elite *Brassica napus. Front. Plant Sci.* 12: 749491. doi:10.3389/fpls.2021.749491.

Xiang Neik, T., Ghanbarnia, K., Ollivier, B., Scheben, A., Severn-Ellis, A., Larkan, N.J., Haddadi, P., Fernando, D.W.G., Rouxel, T., Batley, J., Borhan, H.M., and Balesdent, M. 2022. Two independent approaches converge to the cloning of a new *Leptosphaeria maculans* avirulence effector gene, *AvrLmS-Lep2. Mol. Plant Pathol.* 23: 733–748. doi:10.1111/mpp.13194.

Yadav, S., Modi, P., Dave, A., Vijapura, A., Patel, D., and Patel, M. 2020. Effect of Abiotic stress on crops. In: M. Hasanuzzaman, M. Carvalho Minhoto Teixeira Filho, M. Fujita, and T. Assis Rodrigues Nogueira, eds. Sustainable Crop Production. London, IntechOpen. doi:10.5772/intechopen.88434.

Yang, G., Jiang, X., and Yang, H. 2002. Development of novel pesticides based on phytoalexins: Part 2. Quantitative structure-activity relationships of 2-heteroaryl-4-chromanone derivatives. *Pest. Manag. Sci.* 58: 1063–1067. doi:10.1002/ps.584.

Yu, F., Gugel, R.K., Kutcher, H.R., Peng, G., and Rimmer, S.R. 2013. Identification and mapping of a novel blackleg resistance locus *LepR4* in the progenies from *Brassica napus* × *B. rapa* subsp. sylvestris. *Theor. Appl. Genet.* 126: 307–315. doi:10.1007/s00122-012-1919-2.

Yu, F., Lydiate, D.J., and Rimmer, S.R. 2005. Identification of two novel genes for blackleg resistance in *Brassica napus*. *Theor. Appl. Genet.* 110: 969–979. doi:10.1007/s00122-004-1919-y.

Zheng, X., Koopmann, B., Ulber, B., and von Tiedemann, A. 2020. A global survey on diseases and pests in oilseed rape- current challenges and innovative strategies of control. *Front. Agron*. doi:10.3389/fagro.2020.590908.

# Summary

*Leptosphaeria maculans*, a phytopathogen causes blackleg disease, is an economically significant biotic stress that affects oilseed rape (*B. napus*) production worldwide. Blackleg disease can be controlled by stubble management, crop rotation, fungicides, biocontrol agents, and resistant cultivars. However, until now the latter has been the most effective and sustainable method applied. Therefore, we focused in this study on different aspects of *B. napus* resistance to blackleg disease. Resistance to *L. maculans* infection in oilseed rape can be qualitative or quantitative. Qualitative resistance is mainly favoured for its complete effect while quantitative resistance is preferred for its durability and wide-spread effects against new emerged races.

The first objective of this study was to characterise the races of *L. maculans* in Germany to evaluate the efficacy of blackleg race-specific major resistance (*R*) genes in *B. napus*. Due to the natural evolution of the pathogen, major *R* genes typically go through a “boom and bust” cycle and might lose efficacy a few years after their commercial release. Hence, races of *L. maculans* should be monitored on a regular basis. This provides the farmers with the necessary information that enable them to effectively control blackleg disease in the field. In this study, 574 isolates were collected in different regions in central and northern Germany. The avirulence profile of *L. maculans* population was defined either by pathogenicity tests on cotyledons of a differential set of oilseed rape or by amplifying avirulence gene-specific PCR markers. Ten avirulence genes were characterised in the isolates, namely, *Avrlm1*, *Avlm2*, *Avrlm3*, *Avrlm4*, *Avrlm6*, *Avrlm7*, *Avrlm11*, *LepR1*, *LepR2* and *LepR3*. Results showed that the *Avrlm6, -7, -11, AvrlepR1, -R2* was the most dominant race, and thus, the corresponding major *R* genes *Rlm6*, *Rlm7*, *Rlm11*, *LepR1* and *LepR2* were the most effective *R* genes in the German experimental field. In contrast, *Avrlm2* was absent in all races, highlighting that the major *R* gene *Rlm2* is 100% ineffective in Germany. The frequencies of *Avrlm1*, *Avrlm3*, *Avrlm4* and *AvrlepR3* were less than 42%. This demonstrated that the corresponding major *R* genes were partially effective in the fields explored. The ratio of *L. maculans* mating types *MAT1-1* and *MAT2-1* in the collection was also investigated. For that, a multiplex PCR assay was used to show that the mating types did not deviate from the ratio 1:1 in any of the regions examined. It was concluded, according to the random mating hypothesis, that the sexual outcrossing occured randomly and was dominant in the *L. maculans* populations studied. This is a factor that enhances the emergence speed of new *L. maculans* races.

As major *R* genes might lose their efficacy a few years after commercialisation, a further aim of our study was to seek new sources of quantitative resistance in *B. napus* or its cruciferous relatives. For that, this project aimed to establish a reliable and repeatable inoculation method

that enables a fast but effective greenhouse screening of sources of quantitative resistance. Four inoculation methods were compared in the greenhouse. Results showed that inoculation with a mycelial agar plug on an injured stem base was more reproducible and more accurately differentiated the variances in quantitative resistance to blackleg disease than inoculation with spore suspension on lamina, or inoculation with a mycelial agar plug on an injured petiole. Through stem-based inoculation, 354 DH lines were assessed for quantitative resistance to blackleg disease. The plant material investigated provided a wide genetic background, as it was produced from an interconnected multiparent mapping population generated from six elite varieties with quantitative resistance and one susceptible parent. By monitoring the volume of diseased tissues (VDT), it was clear that quantitative resistance of the DH lines distinctly differed. These results were used for a genome-wide association study that revealed eight significant QTLs which contributed towards 3 to 6% of the phenotypic variation in the mapping population. Although the greenhouse experiments were conducted in parallel to field trials, abiotic and biotic stresses severely hampered the success of the experiments in the field. Drought, insect damage and field raiding by wild boar were the main problems in our phenotyping studies for quantitative resistance against blackleg disease in the field.

Furthermore, this study aimed to evaluate quantitative resistance to blackleg disease in new allohexaploid hybrids (2n= 6X= AABBCC). These hybrids were produced in Giessen University through embryo rescuing followed by chromosome doubling of a triploid (2n=3x=ABC) produced from crossing *B. napus (2n=2x= AACC)* X *B. nigra (2n=2x= BB)*. By comparing the volume of diseased tissue at the stem bases of the parental genotypes and the hybrids, we concluded that an inherited resistance originated from the B genome of *B. nigra* was shown in the hybrids. Further breeding studies should aim to solve the instability problems of the genome combination in the crosses to allow this new resistance source to be used.

An additional objective of the study was to investigate plant chemical defences in the pathosystem *L. maculans- B. napus*. For this experiment, phytoalexin accumulation was checked in a genotype with quantitative resistance and another without. Our analysis, using an HPLC-system combined with high-resolution Q-TOF mass spectrometry, revealed that caulilexin C existed in *B. napus* as a phytoanticipin before the infection with *L. maculans*. On the contrary, rutalexin, spirobrassinin, cyclobrassinin, and brassilexin were induced de novo upon the infection as phytoalexins. The resistant genotype clearly accumulated more phytoalexins than the susceptible one at 7 dpi while the differences were less at 14 and 21 dpi. However, spirobrassinin, which cannot be detoxified by *L. maculans*, was the highest accumulated phytoalexin at all time points in both genotypes. The experimental results showed that there is a correlation between resistance and the accumulation of the above-

mentioned chemical defences. Further studies are needed to assure whether a causative connection exists.

All in all, the results of this study pave the way for agricultural consultants to establishment strategies that preserve the commercialised major *R* gene efficacies in German regions. However, the study emphasized the importance of pyramiding major *R* genes with quantitative resistance to expand their efficacy. This work also provides breeders with potential new sources of quantitative resistance that either have been found in the interconnected multiparent mapping population investigated or from the B- genome of *B. nigra*. Additionally, this research opens the door for more studies on chemical defences induced in the *L. maculans*- *B. napus* pathosystem that can be used in breeding programmes or the agrichemical industry.

# Zusammenfassung

*Leptosphaeria maculans*, ein Phytopathogen, das die Rapskrankheit Phoma Wurzelhals und Stängelfäule verursacht, ist ein wirtschaftlich bedeutsamer, biotischer Stressfaktor, der die Produktion von Raps (*B. napus*) weltweit beeinträchtigt. Dieses Pathogen kann hauptsächlich durch Stoppelmanagement, Fruchtfolge, Einsatz von Fungiziden, Nützlinge und Anbau von resistenten Sorten bekämpft werden. Die Nutzung von Resistenzen gegenüber Phoma stellt jedoch eine effiziente und ökologisch vorteilhaftere Alternative zu anderen Methoden dar. Daher konzentrierten wir uns in dieser Studie auf die Untersuchung verschiedener Aspekte der Resistenz von *B. napus* gegen den Wurzelhals und die Stängelfäule. Resistenzen gegenüber Phoma werden durch qualitative Major-Resistenz-Gene oder quantitative Trait Loci vermittelt. Qualitative Resistenz wird hauptsächlich wegen ihrer vollständigen Wirkung bevorzugt, während quantitative Resistenz wegen ihrer Dauerhaftigkeit und weitverbreiteten Wirkung gegen neu entstandene Rassen bevorzugt wird.

Das erste Ziel dieser Studie war es, die Rassen von *L. maculans* in Deutschland zu charakterisieren, um die Wirksamkeit der rassenspezifischen Major Resistenz (R) Gene gegen Phoma in *B. napus* zu bewerten. Aufgrund der natürlichen Evolution des Pathogens durchlaufen die Major R Gene typischerweise einen „Boom and Bust“-Zyklus und können einige Jahre nach ihrem kommerziellen Einsatz an Wirksamkeit verlieren. Daher sollte regelmäßig ein Rassenmonitoring von *L. maculans* durchgeführt werden. Dadurch erhalten die Landwirte die notwendigen Informationen, die es ihnen ermöglichen, die Phoma Wuzelhals und Stängelfäule auf dem Feld effektiv zu bekämpfen. In dieser Studie wurden 574 Isolate des Pilzes in verschiedenen Regionen Mittel- und Norddeutschlands gesammelt. Das Avirulenz-Profil der *L. maculans* Population wurde entweder durch Pathogenitätstests an Keimblättern eines Raps -Sortiments oder durch Amplifikation von Avirulenzgen-spezifischen PCR-Markern definiert. Zehn Avirulenzgene wurden in den Isolaten charakterisiert, nämlich *Avrlm1*, *Avlm2*, *Avrlm3*, *Avrlm4*, *Avrlm6*, *Avrlm7*, *Avrlm11*, *LepR1*, *LepR2* und *LepR3*. Die Ergebnisse zeigen, dass die dominierenden Rassen dieser Studie Träger der Avirulenzgene *Avrlm6*, *-7*, *-11*, *Avrlepr1*, *R2* sind. Somit sind die zugehörigen Resistenzgene die zurzeit wirksamsten Major *R* Gene im deutschen Rapsanbau. Im Gegensatz dazu fehlte *Avrlm2* in allen Rassen, was zeigt, dass das Major R Gen *Rlm2* in Deutschland zu 100 % unwirksam ist. Die Häufigkeit von *Avrlm1*, *Avrlm3*, *Avrlm4* und *AvrlepR3* in *L. maculans* aller untersuchten Populationen betrug weniger als 42 %. Dies zeigte, dass die entsprechenden Major *R* Gene in den untersuchten Regionen nur teilweise wirksam waren. Das Verhältnis der *L. maculans* Kreuzungstypallele *MAT1-1* und *MAT1-2* in der Isolaten-Sammlung wurde ebenfalls geprüft. Die Resultate zeigen anhand eines Multiplex-PCR-Test für die Kreuzungstypallele *MAT1-1*

und *MAT1-2*, dass im Feldmaßstab keine signifikanten Abweichungen vom 1:1-Verhältnis in der Population an allen Standorten beobachtet werden konnte. Es kann daher von einer bedeutsamen Rate sexueller Rekombination ausgegangen werden, was die Anpassung der Population des Pathogens an im Anbau befindliche aber auch neue Major *R* Gene begünstigen dürfte.

Da die Major *R* Gene einige Jahre nach ihrem kommerziellen Einsatz ihre Wirksamkeit verlieren können, war ein weiteres Ziel unserer Studie die Suche nach neuen Quellen quantitativer Resistenz in *B. napus* oder in seinen verwandten Spezies. Darum zielte dieses Projekt zunächst darauf ab, eine zuverlässige und wiederholbare Inokulationsmethode für das Screening von Pflanzenmaterialien für quantitative Resistenz im Gewächshaus zu etablieren, die schnell und effektiv ist. Im Gewächshaus wurden vier Inokulationsmethoden verglichen. Die Studie zeigt, dass die Inokulation mit einem pilzbewachsenen Agarplug auf eine verletzte Stängelbasis wesentlich reproduzierbarere und stärker differenzierende Ergebnisse lieferte als dies für die Inokulation der verletzen Petiole mit einem pilzbewachsenen Agarplug oder die Inokulation der Blattober- oder –unterseite mit einer Sporensuspension der Fall war. Anhand der Inokulation der Stängelbasis mit einem pilzbewachsenen Agrarplug wurden 354 Raps DH-Linien auf quantitative Resistenz gegen Phoma untersucht. Das untersuchte Pflanzenmaterial lieferte einen vergleichsweise breiten genetischen Hintergrund, da es aus einer Interconnected-Multiparent Kartierungspopulation bestand, die aus sechs Elitesorten mit einem anfälligen Elter generiert wurde. Durch die Bonitur des Volumens erkranktem Gewebes an der Stängelbasis (Volume of Diseased Tissue, VDT) wurde gezeigt, dass sich die DH-Linien hinsichtlich ihrer Resistenz signifikant unterschieden. Diese Ergebnisse wurden für eine genomweite Assoziationsstudie verwendet, die acht signifikante QTLs ergab, die 3 bis 6% der phänotypischen Variation in der Kartierungspopulation erklären. Obwohl die Gewächshausversuche parallel zu Feldversuchen durchgeführt wurden, beeinträchtigten abiotische und biotische Stressfaktoren den Erfolg der Feldversuche stark. Dürre, Insektenschäden und Feldüberfälle durch Wildschweine waren die Hauptprobleme für unsere Phänotypisierungsstudien zur quantitativen Resistenz gegen die Phoma Wurzelhals und Stängelfäule im Feld.

Darüber hinaus zielte diese Studie darauf ab, die quantitative Resistenz gegen Phoma bei neuen Allohexaploiden Hybriden (2n = 6X = AABBCC) zu bewerten. Diese Hybriden wurden an der Universität Gießen durch „Embryo Rescue“ mit anschließender Chromosomenverdopplung eines Triploiden (2n=3x=ABC) hergestellt, das aus der Kreuzung von *B. napus* (2n=2x= AACC) X *B. nigra* (2n=2x= BB) hervorgegangen ist. Durch den Vergleich des Volumens des erkrankten Gewebes (VDT) an der Stängelbasis der elterlichen Genotypen und der Hybride schlossen wir, dass in den Hybriden eine Resistenz vererbte

wurde, die aus dem B-Genom von *B. nigra* stammte. Weitere wissenschaftliche Studien zur Lösung des Problems der Instabilität der Genomkombination in den Kreuzungen sollten durchgeführt werden, um die Nutzung dieser neuen Resistenzquelle zu ermöglichen.

Ein weiteres Ziel der Studie war die Untersuchung der pflanzenchemischen Abwehr im Pathosystem *L. maculans*- *B. napus*. Für dieses Experiment wurde die Phytoalexin-Akkumulation in einem Genotyp überprüft, der eine quantitative Resistenz zeigte, und einem anderen, der im Gegensatz dazu eine Anfälligkeit zeigte. Unsere Analyse mit einem HPLC-System in Kombination mit Q-TOF-Massenspektrometrie ergab, dass Caulilexin C in *B. napus* bereits vor der Infektion mit *L. maculans* als Phytoanticipin existierte, während Rutalexin, Spirobrassinin, Cyclobrassinin und Brassilexin nach der Infektion als Phytoalexine de novo induziert wurden. Der resistente Genotyp reicherte deutlich mehr Phytoalexine an als der anfällige bei 7 dpi. Bei 14 und 21 dpi waren die Unterschiede geringer. Spirobrassinin, das von *L. maculans* nicht entgiftet werden kann, war jedoch zu allen Zeitpunkten in beiden Genotypen das am höchsten akkumulierte Phytoalexin. Die Ergebnisse dieser Experimente zeigten, dass es eine Korrelation zwischen der Resistenz und der Akkumulation der erwähnten chemischen Abwehrstoffe gibt. Weitere Untersuchungen sind erforderlich, um sicherzustellen, ob ein ursächlicher Zusammenhang besteht.

Die Ergebnisse dieser Studie ebnen landwirtschaftlichen Beratern den Weg, Strategien zu etablieren, um die Wirksamkeit der kommerzialisierten Major *R* Gene in deutschen Felder zu erhalten. Die Studie betont jedoch die Bedeutung der Pyramidisierung der Major *R* Gene mit quantitativer Resistenz, um ihre Wirksamkeit zu erweitern und die lebensdauer zu verlängern. Diese Arbeit liefert den Züchtern auch neue potenzielle Quellen für quantitative Resistenz sowohl aus der untersuchten Interconnected-Multiparent-Kartierungspopulation als auch aus dem B-Genom von *B. nigra*. Es öffnete zudem die Tür für weitere Studien zu chemischen Abwehrmechanismen, die im *L. maculans*- *B. napus* Pathosystem induziert werden und in Zuchtprogrammen oder in der Agrarindustrie verwendet werden können.

# Acknowledgements

At the end of this journey I would like to express my deepest gratitude to my parents, Huda Alyoubi and Yasir Alnajar, who always encouraged me and sacrificed a lot to pave the way for me to develop. I thank them from all my heart for their dedication and for believing in me. Their love and words were always my candles in the difficult moments.

I would like to express my deepest appreciation and gratitude to my supervisors Dr. Birger Koopmann and Prof. Andreas von Tiedemann for giving me the opportunity to do my PhD under their supervisions. The completion of this thesis could not have been possible without their guidance and support. I cordially thank Dr. Birger Koopmann for all the scientific discussions that deepened my skills and understanding of phytopathology along the PhD journey. Furthermore, I appreciate his kindness and guidance on personal level. I am extremely grateful to Prof. von Tiedemann for his great support to solve problems and for his scientific feedback and recommendations that helped me to improve and learn a lot. I was always inspired by his enthusiasm about crop protection since I have been master student under his supervision. I am greatly indebted to Prof. Petr Karlovsky, Dr. Mohammad Alhussein, Dr. Christian Obermeier, Paul Vollrath, Dr. Annaliese S. Mason and Dr. Roman Gaebelein for the fruitful and good cooperation in this project. I'm extremely grateful to Prof. Anna-Katrin Mahlein for being a member in my thesis committee.

I wish to extend my special thanks to all staff members of the wonderful team in Division of Plant Pathology and Crop Protection. Many thanks go to Jutta Shaper, Heike Rollwage, Isabel Müller, Dagmar Tacke, Evelin Vorbeck for their indispensable technical support and for making the work more enjoyable. My sincere thanks go to Hubertus Reintke and Maik Knobel for their support in field sampling. I also would like to show my gratitude to all the students that took part in this project. My great thanks go to Martina Bode for her efficient assistance with bureaucratic issues.

Many thanks to Dr. Susanne Weigand for accepting me in the master program Crop Protection and supporting me to overcome difficulties faced me to travel to start my master study in Göttingen till I started my first steps in Göttingen. Her kindness and support along these years are priceless. My deepest gratitude is extended to Dr. Anke Sirrenberg for her support at scientific level and personal level. It was always great to be in Anke´s class as a student at the beginning and as assistant later. Many thanks go to Dr. Jean Wagner for all kind of support he gave and for being a great friend.

I am also thankful for my classmates and friends  Sarenqimuge Sarenqimuge, Yao Wang, Musrat Surovy, Rebecka Dücker, Marta Vega Marin, Barbara Navarro, Annette Pfordt, Sebastian Streit for their encouragement, support, as well as the great memories together. I would like to extend my thanks to William Nelson for proof-reading the thesis and being very patient and sympathetic.

On this occasion I would like to express my big acknowledgment to the collectives PVW and MLK with whom I discover again and again the power and wisdom of the “oak tree”. Also, I extend my thanks to all friends in “Happy Göttingen” and “Sobremensa” groups who filled those years with joy and happiness. There are no words to express my thanks to my special friends to whom I have never needed to explain myself: Mohannad, Dima, Ameen, Peter, Karin, Lars and Sebastian. Discussions with them was essential during the way to re-balance, to gain determination and optimism. Many thanks goes to Dana Gaef for his kindness and help to tackle several obstacles that faced me during this journey.

I would be remiss in not mentioning my brother Wisam, who appeared always with creative solutions for things that seems to be impossible. I thank him for his love and support. There is no words to express my love and gratitude for Syria where I studies 26 years. I thank deeply all my teachers there for all their inspiration and for motivating me to love science.

Lastly, I'd like to thank my University: Georg-August-Universität Göttingen. I was honored to be a master and PhD student here. I also thank the Federal Ministry for Food and Agriculture in Lower Saxony, Germany for funding my project.

# Statutory declaration

I hereby declare that this dissertation was undertaken independently and without any unaccredited aid.

Göttingen, 08th July 2022

Dima Alnajar